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# Chemotaxonomic Exploration of Fungal Biodiversity for Polyketide Natural Food Colorants…

Discovery & evaluation of cell factories, and characterization of pigments

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Ph.D. Thesis

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# Chemotaxonomic Exploration of Fungal Biodiversity for Polyketide Natural Food Colorants…

Discovery & evaluation of cell factories, and characterization of pigments

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*This thesis is dedicated to the loving memory of my parents.*

## PREFACE

The present thesis is submitted to the Technical University of Denmark (DTU) as a major part of the requirements for the attainment of a PhD degree in Biotechnology. The work presented in the thesis was primarily carried out at Center for Microbial Biotechnology (CMB), Department of Systems Biology, DTU. A few experiments were performed at Research and Development (R&D), and Color Application laboratories of Chr. Hansen A/S, Hørsholm, Denmark. The project was financed by DTU, Chr. Hansen A/S, and the Center for Advanced Food Studies (abbreviated in Danish as LMC) - Food Graduate School. The project work was carried out under the supervision of Docent Dr. Ulf Thrane from CMB, and Professor Dr. Anne S. Meyer from the Department of Chemical and Biochemical Engineering-DTU. The industrial representative collaborators were Vice president (R&D) Dr. Annette Salskov-Iversen and Fungal Specialist Dr. Hans van den Brink.

I always wished to pursue my scientific career in the applied research in multidisciplinary sciences for which I had acquired higher education abroad. The present PhD project happened to me as a “tailor-made” and I thoroughly enjoyed working on it, acquiring and imparting knowledge, entering the challenging fields of color science and color chemistry. There is so much more to learn about this intriguing field of fungi and pigments and I really wish and hope that this thesis would mark a new beginning in this specific area and a constructive reading for people from many different backgrounds. For a layman, the fungal world is alien and hazardous and the work presented in the thesis may be seen as an attempt to bridge the gap between the science and the society by seeking the fungal sources to meet the rising demand of natural food colorants. However, to educate the consumer about the beneficial role these fungi may play for the mankind is beyond the scope of the present thesis but definitely an important issue that needs to be addressed considering the fact that the acceptance of such fungal food colorants, to quite an extent, would eventually be governed by the societal norms.

*Sameer Ahmed S. Mapari*

February, 2009,
Lyngby, Denmark

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It's an overwhelming and gratifying feeling as if a river were to congregate into an ocean or as if a journey has ended with the aimed destination and trying to rest a while before taking a plunge into another journey. But such journeys are always accompanied by numerous people, events, and gestures (articulate or subtle), and I would like to express my heartfelt gratitude to people who have contributed, directly or indirectly, to the successful completion of the present research work.

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# SYNOPSIS

The growing concern over the eventual harmful effects of synthetic colorants on both the consumer and the environment has raised a strong interest in natural coloring alternatives. As a result the worldwide demand for colorants of natural origin is rapidly increasing in the food, cosmetic and textile sectors. Natural colorants can be used for a variety of industrial applications, for instance, as dyes for textile and non-textile substrates such as leather, paper, *etc*., as paints and coatings, in the cosmetic industry, and last but not the least, as food additives. The work presented in this PhD thesis mainly focuses on the food use of natural colorants. The value of the international food colorant market was estimated at around $ 1.15 billions in 2007 (€ 731 million), up 2.5% from $1.07 billion (680 million) in 2004, according to Leatherhead Food International (LFI) [www.foodnavigator-usa.com]. Natural colorants now make up 31% of the colorant's market, compared with 40% for synthetics, according to LFI. Thus, it can be said that naturally derived colorants look set to overtake synthetic alternatives in market value due to the rising demand for the clean label ingredients even though they are relatively expensive to produce. Currently, the vast majority of the natural food colorants permitted in the European Union and the United States are derived by extraction of the pigments from the plant materials obtained from the flowering-plants of the kingdom Plantae and also in one or two cases from insects. Thus, the production of many natural colorants has a disadvantage of dependence on the supply of raw materials, which are influenced by agro-climatic conditions – in addition, their chemical profile may vary from batch-to-batch. Moreover, many of the pigments derived from contemporary sources are sensitive to heat, light, and oxygen, and may even change their colour in response to pH changes. Many ascomycetous fungi naturally synthesize and secrete pigments and thus provide a more reliable source for natural, "organic" food colorants.

The present study forms and proves a scientific rationale towards how to select filamentous fungi as sources of polyketide based natural food colorants considering the enormous biodiversity of fungi and the complexity involved in the biosynthesis of such polyketide pigments. The prime objective of the work presented in this thesis was to test the hypotheses that polyketide pigments produced by filamentous fungi are similar in structure and functionality to those derived by more traditional sources or filamentous fungi can produce known or novel colorants as lead compounds with a potential of improved functionality in food model systems. Finally, it was imperative to evaluate the

production potential of a few of the selected pigment producers as promising fungal cell factories for the future production of polyketide natural food colorants by "green chemistry" concept avoiding use of genetic manipulation.

A cohesive **summary** comprising of a brief description of the proposed research questions of the papers (presented as chapters in this thesis), key findings in relation to the finding of others in the same field with a critical view, and an assessment of the applied methodologies used in the research is presented to start with. The first **two Chapters** of the thesis introduce the subject starting from a broader perspective to the more focused aims and objectives of the project. The **first Chapter** gives **background** information about the topics that find relevance to the essential core of the thesis. The **introductory Chapter** gives an insight to the biodiversity and chemodiversity of fungal pigments and secondary metabolites. The focus is on fungal polyketide class of pigments with an illustration of *Monascus* pigments; well known polyketide pigments of the East Asia. It also throws light on the state-of-the-art knowledge about the biosynthesis and functionality of fungal polyketide pigments. It ends with a review article that summarizes the state-of-the-art of the currently authorized natural food colorants their merits vs. demerits and addresses the issue of how to explore the bio- and-chemodiversity of filamentous fungi to come up with promising cell factories for the production of polyketide natural food colorants. The rest of the **five Chapters** form the experimental work of the thesis and are supplemented with already published or submitted journal articles. Finally, cohesive conclusions and future perspectives are presented together with a resume that highlights the key contents of the thesis.

The first and the foremost requirement towards selection of potential pigment(s) producers is that the potential fungus producing the pigment(s) is non-toxigenic under the given broader range of production conditions and is non-pathogenic to humans. The ability of filamentous fungi to co-produce mycotoxins along with industrially useful extrolites, e.g. as in case of citrinin produced by some of the pigment and statins producing *Monascus* species, is a major bottleneck in their approval by the legislative authorities. Some of the pigment producers, for instance, *Penicillium marneffei*, could even be human pathogens. In the light of this, **Chapter 3** addresses the question of how to rationally select and identify promising fungal pigment producers – meeting the above-mentioned requirement - considering the enormous chemical and biodiversity of fungi. Also the fact that the molecular and genetic basis for the polyketide pigment biosynthesis in fungi have not yet

been examined in detail, makes genomic approaches for screening unfeasible at this point of time. The message was to prove our hypothesis that the pertinent use of chemotaxonomic trait of metabolite profiling using tools such as HPLC-DAD-MS and *a priori* knowledge of fungal extrolites is a rational approach towards selection of potentially-safe polyketide natural colorant producing fungal cell factories that are neither known to be human pathogens nor to produce any known mycotoxins. The findings are reported in **Chapter 3**.

The structures of polyketides often contain polyunsaturated functionality exhibiting characteristic UV-vis spectra. Therefore, they may serve a potential source of new chromophores (color leads) that can be promising as food colorants. We hypothesized that the hues and chroma of the pigments from chemotaxonomically selected ascomycetous fungi in the red and the yellow spectra are comparable to the existing natural food colorants. In the light of this, the color characteristics of the pigment extracts from chemotaxonomically preselected ascomycetous fungi were compared to natural colorants currently in use in CIELAB color space using quantitative colorimetry (**Chapter 4**). The work presented in **Chapter 4** has shown that there is a great potential in the pigment producing genera in addition to *Monascus* that produce colors with similar hues and chroma to those of commercially available colorants in the yellow spectra. Especially the color hues of *Epicoccum nigrum* extracts were markedly similar to the color hue of turmeric; a commercially available colorant from the rhizome of the plant *Curcuma longa*. The pigment extract of *E. nigrum* IBT 7901 on YES medium was shown to contain a predominant colored component identified as a polyketide, orevactaene. Also, colorants from fungi such as *Penicillium purpurogenum* IBT 11180 and *P. aculeatum* IBT 14263 in addition to *Monascus* colorants were shown to provide additional hues in the red spectra. Pigment extract of *Penicillium purpurogenum* IBT 11180 on YES medium was shown to contain PP-R, a derivative of *Monascus* pigment monascorubramine. The above-mentioned findings form the subject matter of **Chapter 4**. Strains of *Epicoccum nigrum* as yellow pigment producers and strains of *P. purpurogenum* and *P. aculeatum* as orange-red pigment producers were considered for further study.

*Monascus* pigments have been used as natural food colorants in Asia for centuries. They are not authorized for use in the European Union and the United States mainly due to the risk of co-production of the mycotoxin citrinin by *Monascus* spp. It is a quite known fact that the polyketide pathway, leading to the synthesis of the colored azaphilone compounds

like *Monascus* pigments is widely distributed in the fungal world. Therefore, we hypothesized that *Monascus* or Monascus-like azaphilone pigments are likely to be produced by strains of the species in the *Penicillium* subgenus *Biverticillium* that produce copious amounts of yellow, orange, and red pigments and includes species such as *P. purpurogenum* one of the strains of which (IBT 11180) was found to produce a *Monascus* pigment derivative (**Chapter 4**). **Chapter 5** reports on the screening for novel producers of *Monascus*-like pigments in *Penicillium* subgenus *Biverticillium*. The screening was carried out using the X-hitting algorithm as a chemotaxonomic tool which searched for the most similar UV-vis spectra of the metabolites present in the pigment extracts to those of the selected reference metabolites (*Monascus* pigments and citrinin). It has also been reported that *Monascus* pigments such as monascin and rubropunctatin form the same biogenic family, which might include such mycotoxins as citrinin. Therefore, the strains were analyzed for the absence of citrinin**.** *Monascus* pigments were discovered in the extracts of two closely related species of *Penicillium* that were only distantly related to the genus *Monascus*. Monascorubrin, xanthomonasin A and threonine derivative of rubropunctatin - were identified in the extract of *Penicillium aculeatum* IBT 14263 and monascorubrin was identified in the extract of *Penicillium pinophilum* IBT 13104. None of the tested *Penicillium* extracts showed the presence of citrinin. These findings are reported and discussed in **Chapter 5**.

The variation in the photostability among the currently authorized natural pigments limits their application span to a certain type of food system that “fit” the stability requirements of the colorant. Hence, there is a great scope for the discovery of relatively more stable natural pigments that may be used for a wide industrial application without the potential use of formulation techniques. Polyketide pigments such as *Monascus* pigments are known to be unstable towards UV and the visible light compared to their amino acid derivatives. Also not much is known about the chemistry behind the color change of fungal polyketide pigments including *Monascus* pigments in food systems that are exposed to light. Therefore, a study was undertaken (**chapter 6**) with the purpose of investigating the light stability of an orange-red and a yellow promising fungal pigment extract from *P.aculeatum* IBT 14263 and *E. nigrum* IBT 41028 respectively in a soft drink model medium and in citrate buffer at low and neutral pH. Natural colorants including fungal polyketide pigments are (in most cases) a mixture of several components and therefore it would be interesting to see the extent of stability of individual components

towards light. We hypothesized that some of the components could be more photostable than the others and that their discriminative destabilization would affect the resultant colour before and after the light exposure. The quantitative and qualitative color change pattern of the fungal pigment extracts indicated the enhanced photostability of fungal pigment extracts compared to the commercially available natural colorants *Monascus* Red and turmeric used as controls. Yellow components of the orange-red fungal pigment extract were more photostable than the red components. Chemistry of photodegradation of the two pigment extracts was studied by HPLC-DAD-MS. This forms the subject matter of **Chapter 6**.

Secondary metabolite producing ability of filamentous fungi is facilitated on solid substrates as they are the natural habitat for most fungal species. This has triggered the interest in solid substrate fermentation (SSF) for a range of fungal secondary metabolites. It has also been shown that solid media like rice supports higher pigment yield in case of *Monascus* owing to better hyphal adhesion and penetration offered by the microstructure of the rice grain. On the other hand, the industry appears to favor the use of submerged liquid fermentation (SLF) owing to its easier product recovery and purification, and the use of standardized conditions in terms of culture media choice and typical fermentation parameters. Therefore, to explore the possibility of pigment production in a variety of liquid media forms a necessary step towards the large scale industrial set up. We studied both of these possibilities with the selected pigment producers (strains of *Epicoccum nigrum*) in **Chapter 7**. Attempts were made to study the micro and the macro environment around the production organism; morphology – if a particular morphology favours the pigment production, the choice of substrate that can be manipulated to get the maximum pigment production. We tested the hypothesis that the use of buffer can reduce the pellet formation in the liquid media. The findings are reported in **Chapter 7. Chapter 7** also brings out the potential of a few Penicillia for orange-red pigment production in liquid media with or without solid support for the mycelia and the possibility of “tailor-made” colorants.

## Dansk sammenfatning

Kemotaksonomisk undersøgelse af svampebiodiversitet til naturlige polyketider som farvestoffer til levnedsmidler: Opdagelse og evaluering af cellefabrikker og karakterisering af pigmenter.

Naturlige farvestoffer vil i fremtiden udgøre en større markedsværdi end syntetiske farvestoffer på grund af producenternes fortsatte stræben efter at imødekomme det stigende krav om rene og sikre ingredienser - særligt til levnedsmiddelindustrien. Store dele af den nuværende produktion af godkendte naturlige farvestoffer, hvoraf de fleste kommer fra planter og/eller insekter, har flere ulemper bl.a. at tilgængeligheden af råmaterialer er afhængig af agro-klimatiske forhold. Mange skimmelsvampe danner naturligt farvestoffer, som de udskiller til omgivelserne, og er på derfor et pålideligt alternativ, og/eller en ny ressource, til naturlige farvestoffer til levnedsmidler. De svampeproducerede naturlige farvestoffer kan fremstilles med stort udbytte ved at benytte optimale dyrkningsteknologier, og dette kan sikre en effektiv farvestofproduktion uden brug af genetisk manipulerede organismer. Svampepigmenterne er sekundære metabolitter, hvoraf mange er strukturelt meget forskellige polyketider, der syntetiseres af multifunktionelle enzymer, også kaldet polyketid syntaser. Eftersom den molekylære og genetiske basis for polyketid pigmenterne endnu ikke er udforsket og kendt i detaljer, er det ikke muligt at benytte en genetisk tilgang for udforskningen af svampepigmenter og derfor blev svampebiodiversiteten udforsket med en kemotaksonomisk tilgang. Gennem dette blev flere potentielt sikre og lovende pigmentproducenter identificeret, mens mykotoksinproducerende og/eller patogene pigmentproducenter aktivt blev fravalgt. Svampepigmenternes farve blev karakteriseret og det er påvist at der er skimmelsvampeslægter der kan danne farvestoffer i det røde og gule spektrum der er sammenlignelige med de kommercielt tilgængelige naturfarvestoffer. I Asien er polyketid farvestoffer fra *Monascus* meget brugt, men disse er ikke tilladte i USA eller Europa på grund af risikoen for en samtidig produktion af et mykotoksin, citrinin. På denne baggrund er der et øget behov for at lede efter nye og sikre producenter af poyketid pigmenter, og der blev fundet ti nye lovende producenter af *Monascus*-lignende farvestoffer i *Penicillium* slægten, der samtidig ikke var i stand til at danne citrinin. To repræsentative pigmentekstrakter (et orange-rødt og et gult) viste sig at være mindre lysfølsomme i levnedsmiddelmodelsystemer ved sammenligning med et kommercielt *Monascus* farvestof og gurkemeje. Endelig blev en af farvestofproduktionsstammerne dyrket på flydende substrater og på et fast rissubstrat, og vurderingen af vækst, myceliemorfologi og produktion af farvestoffer viste klart at denne skimmelsvamp er en potentiel kandidat som cellefabrik til produktion af naturlige polyketider, der kan benyttes som farvestoffer til levnedsmidler.

**SUMMARY**

Even though filamentous fungi have been known to produce pigments with extraordinary range of colours and chemical classes since ages, pigments have been used mainly as a taxonomic tool for identification and species differentiation until the start of the ongoing decade. Barring the example of carotenoids, the use of filamentous fungus as a source of natural food colorant is limited to *Monascus* pigments (polyketide based) that too only in the South East Asia. On the other hand, the demand for natural colorants has been growing in the recent years owing to the healthier lifestyles of consumers. The production system of natural food colorants is based on the supply of agricultural based raw materials which is dependent on the climatic conditions and thus results in batch-to-batch variation of the extracted pigment profile. This has fuelled research to explore the alternative and/or additional route for the production of natural food colorants through the application of biotechnological tools to microorganisms including filamentous fungi.

The work presented in the present thesis is a proof of concepts towards the potential use of filamentous fungi other than *Monascus* for polyketide water-soluble pigments and the tremendous potential of such pigments as food colorants compared to the existing natural colorants derived from contemporary sources. The research done was much applied and industrially relevant. The most significant contribution of the present research is bringing out the potentially safe future cell factory possibilities of filamentous fungi that produce polyketide pigments naturally without the use of genetic manipulation techniques. A patent application on the production of *Monascus*-like polyketide based azaphilone pigments in Penicillia is already applied.

Following are some of the proposed research questions addressed in the thesis (**Chapter wise**) with the most significant findings and an assessment of the applied methodologies:

The challenging question in the present research project (**Chapter 3**) was how to approach towards the selection of a promising pigment producer considering the enormous chemo- and bio-, diversity of filamentous fungi. Also that in the so-called genomic era it is still not much known about the genetic basis of the biosynthesis and regulation of these complex polyketide pigments (being secondary metabolites) involving multistep fungal iterative type I polyketide synthases (PKS). Therefore, instead of a genetic approach, classical disciplines such as chemotaxonomy, biochemistry and microbial physiology were used. Filamentous fungi have been known to produce secondary metabolites, the profiling of which has been central in the development of fungal systematics, taxonomy, and ecology

for more than 25 years. However, what is significant and exemplified in this thesis is that the appropriate use of metabolite profiling integrated in a multidisciplinary approach to applied mycology can be quite handy in the discovery of leads as pigments. Such an approach with a focus on metabolite profiling using chemotaxonomic tools has been shown to successfully explore and exploit the ascomycetous fungi as potentially safe cell factories for the future production of polyketide natural food colorants. A comprehensive list, based on the chemotaxonomy, of a majority of the polyketide pigment producing ascomycetous fungi, their pigment composition, the toxigenic potential with *a priori* knowledge of metabolite profiles of known (coloured as well as uncoloured) toxic metabolites, and pathogenic potential on the basis of BioSafety Level (BSL) classification is provided. This chemotaxonomic rationale formed the basis of our pre-selection/deselection of pigment producers. This could also be very handy for the selection of potentially safe fungal cell factories not only for polyketide pigments but also for the other industrially important polyketides; the molecular and genetic basis for the biosynthesis of which has not yet been examined in detail.

The color characteristics of a few of the selected potential pigment producers was then analyzed using quantitative colorimetry to emphasize their similarity or differences to the existing commercially available counterparts used as references in the study. Also the pigment composition of some of the representative pigment extracts were analyzed chromatographically, and the key components were identified by high resolution liquid chromatography-diode array detection-mass spectrometry. These points were investigated in **Chapter 4**. Since one of the orange-red pigment extract of a strain (IBT 11180) belonging to *Penicillium purpurogenum* was found to produce a *Monascus* pigment derivative, The question was whether these *Monascus*-like pigments were produced by the other members belonging to the *Penicillium* subgenus *Biverticillium*, a group of Penicillia that were not reported to produce citrinin or any other known mycotoxins but produced copious amounts of yellow, orange and red pigments likely to be polyketide based. The discovery of two novel sources of yellow, orange, and purple-red Monascus-like pigments in *P. aculeatum* IBT 14263 and *P. pinophilum* IBT 13104 without co-production of citrinin **(Chapter 5)** is a milestone of the thesis that opened the door to look for more promising sources of such polyketide natural colorants in the other genera including Penicillia. The significance of the discovery of such potentially safe cell factories also lies in the fact that these *Monascus* pigments have been consumed in the East Asia for

hundreds of years without a major report of their health hazards. Thus, it can be said that they are safe to be consumed in a proper dosage and their production in a safer host would form a new landmark in the future biotechnological production of these natural food colorants. *Monascus*-like pigments have been reported to be produced by an unidentified species of *Penicillium* (see **Chapter 5** reference no. 36), but the chemotaxonomic approach in the present study draws attention to the correct identification of the culture, its cultural and physiological characteristics; functional features of fungi that is of major interest to the biotechnological industry, but often de-emphasized in the discovery of leads including pigments.

The next question was whether such polyketide pigments are stable in food systems that could be of high relevance for their application in broader range of food products. This much applied issue was addressed in **Chapter 6** whereby the photostability of 2 promising pigment extracts was studied considering the reports that indicated that *Monascus* pigments are prone to photo degradation. Majority of the earlier reports on the photostability of *Monascus* pigments did not use any benchmarks for comparison and the spectra and the intensity of light used for the experiment was not realistically interpreted. The findings presented in **Chapter 6** were more realistic indicating that *Monascus* pigments may not be as unstable towards light as some of the currently used colorants but the two pigment extracts under study were found to be more light-stable than the *Monascus* pigments. The reason for a better light-stability, for the red pigment extract, was that it included *Monascus* pigment derivatives which are more light-stable than their counter parts (for detailed description see **Chapter 6**) This was in agreement with the findings of Jung *et al.* (see reference no. 8 in **Chapter 6**).

The questions, whether the selected pigment producer was able to produce pigments in the liquid media, if key carbon and/or nitrogen sources would result in a different pigment profile, and if a particular morphology favours pigment production, were addressed in **Chapter 7**. The selected pigment producers were *Epicoccum nigrum* and *Penicillium purpurogenum*. Among the four tested strains of *E. nigrum*, *E. nigrum* IBT 41028 exhibited a broader pigment producing ability in a variety of liquid media, produced 4.6-fold more pigments in the liquid unoptimized medium compared to *Monascus ruber* IBT 7904 used as control but produced 1.6-fold lower pigments on rice compared to the control. This study is a first ever study on the comparative production of pigments on rice and liquid media by *E. nigrum* and *M. ruber*. The study brought out the potential of

*Epicoccum nigrum* IBT 41028 as a production organism for polyketide natural food colorants. *P. purpurogenum* IBT 11181 was shown to be a potential pigment producer owing to its tremendous pigment producing ability in the liquid media. The possibility of producing "tailor-made" colorants by manipulating media ingredients was demonstrated and it indicates the future promising perspective of securing such pigments by media optimization avoiding genetic manipulation.

The applied methodologies in the present study involved micro-scale extraction method, use of metabolomic and/or screening tools such as HPLC-DAD-MS and mathematical algorithm X-hitting, colorimetric tools such as UV-vis spectrophotometers, chromameter, and the light cabinet (suntest XLS+). Micro-scale extraction is a well established method and has been used in chemotaxonomy for the quick handling of a large number of fungal isolates grown on solid agar media. Therefore, this method was used for the screening of pigment producers grown on a variety of solid media. The identification tool used in the thesis was LC-DAD-MS. This tool was quick and efficient and a large number of samples could be handled. The LC method used could target a broad range of metabolites, and was reliable, quick and standardised at our research center for several years. For LC-MS mainly positive ESI conditions were used. The presence of compound was detected as the most prominent molecular ion (protonated ion $[M+H]^+$) and confirmed by adducts. The elementary composition calculation was done on protonated ion $[M+H]^+$. The dereplication was performed by combining mass spectra with the UV-vis spectra. In **Chapter 5**, it has been shown that utmost care should be taken when comparing the UV-vis and/or mass spectral data to the discrepancy in the data available in the literature for the identification of colored compounds as this is an important step towards the discovery of new compounds. One example of discrepancy in reporting MS data could be of Jung *et al*. (reference 12 in C**hapter 5**), where $[M + H]^+$ values were shown to be the molecular weight for *Monascus* pigments and their amino acid derivatives. This discrepancy in data was confirmed by calculating molecular weight by the given molecular formula.

Use of the algorithm X-hitting as a screening tool (**Chapter 5**) enabled fast paced (less than 15 minutes) and systematic identification of compounds in crude pigment extracts on the basis of their spectral similarity to the metabolites used as a reference in our study. No false positive results were obtained, some false negative results were encountered that could be explained as in the section *citrinin hits* in (**Chapter 5**). Colorimetric tools and the light cabinet were used as per the guidelines of Chr Hansen A/S.

In conclusion, the work presented in the thesis demonstrates the potential of filamentous fungi and polyketide pigments for future biotechnological production of natural colorants for food applications provided necessary toxicological testings are carried out. In addition, the chemotaxonomic rationale and tool were shown to be effective in screening such potential pigment producers considering the fact that the genomic era is still at a premature stage to predict the functional genes for such polyketide colorants.

**CONTENTS**

# *Chapter 1*

# *Background*

## Background

Colorants impart visual appeal to the substrates to which they are added for the gratification of the human mind and soul. Historically, colorants have been used as dyes to color the fabrics in the textile sector, as paints and coatings to color various inanimate objects, as printing ink, in cosmetics and last but not the least in food stuffs. The history of colorants for food use goes back to the times of Romans where they used saffron and other spices to color the food stuff. Although the present work deals with the colorants that could be used for numerous industrial applications (as mentioned above) but the emphasis is given to the food use in this study. Therefore, the term colorants would mean pigments used in the process of coloring foods throughout the thesis.

Color is a key organo-leptic attribute that provides clues for many food qualities such as flavor, sanity, naturalness or maturity. Based on these clues, consumers make their choices (1). Thus, it can be said that color is a key marketing tool for food. Color is added to food to enrich or compensate for the loss of color during storage or processing of food. It may be added to minimize batch-to-batch variations or to enhance the visual appeal of the otherwise uncolored food. So, the use of food colorants in the food industry is a significant factor for both food manufacturers and consumers in determining the acceptability of processed food (2).

## 1.1 Basic concepts of color

Light is a type of electromagnetic waves and the electromagnetic spectrum visible to human eye lies in the wavelength range of 400-700 nm (Figure 1.1). Humans perceive light of specific wavelengths as colors (3). Color is the visual effect that is caused by the spectral composition of the light emitted, transmitted or reflected by colored objects. It is the result of physical modification of light by pigments. It is observed by the human eye (perception) and interpreted by the human brain (psychology).

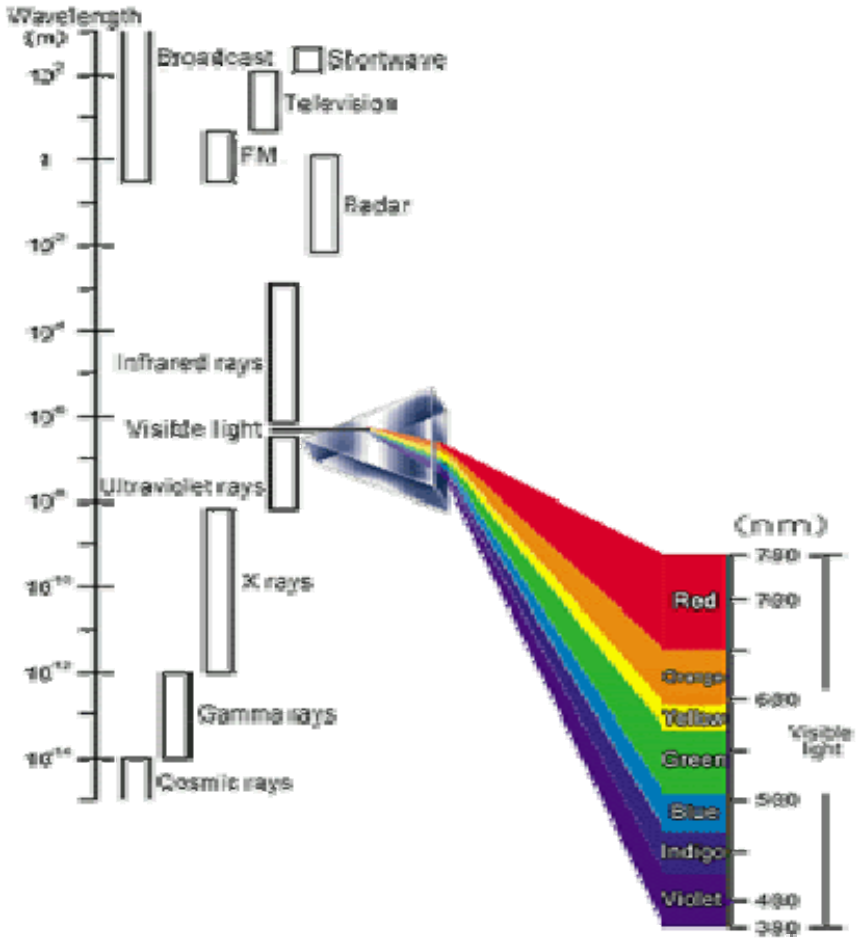



**Figure 1.1 Spectral composition of light (3)**

Thus, color vision is based on a variety of physical, chemical, physiological, and psychological processes. Color vision and colorimetry forms the background of **Chapter 4**, and hence only fundamental aspects of color vision are mentioned below for a quick overview.

The color of an object depends upon the type of light source as it would define the composition of the visible spectrum of the light (**Figure 1.2**), absorbance and/or reflectance of light in the visible range, the observer (human eye or instrument). Same color may look different when viewed in two different light sources, an effect called metamerism (3).

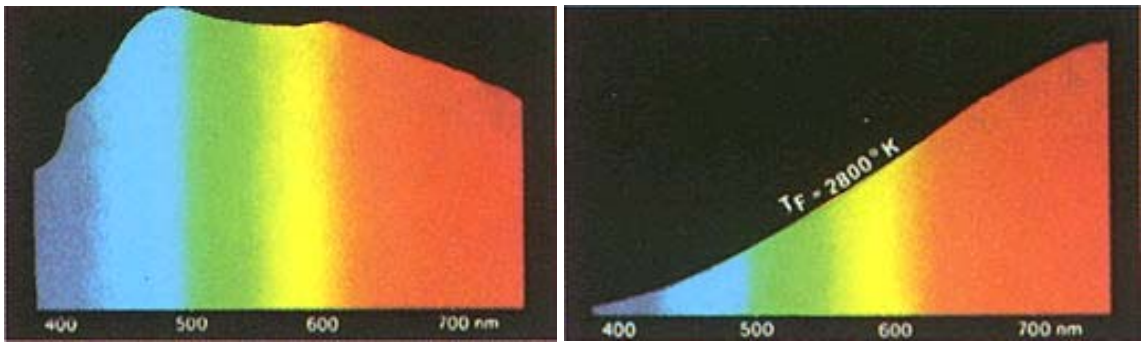



**Figure 1.2 Spectral composition of two light sources (3)**

When matter completely absorbs all the incoming visible light, then the body is visualized as *black*, while complete reflectance of the incoming visible light would render an object *white*. Furthermore, when a constant fraction of the complete visible spectrum of light is absorbed, the absorbing body appears gray. Black, white, and gray are called achromatic colors. Chromatic colors, on the other hand, are generated when matter specifically absorbs (or reflects) light of a given wavelength, which leads to absorption bands with typical maxima and minima in the visible spectrum. For instance, when matter absorbs in the violet part (shorter wavelength) of the visible spectrum (400-430 nm), that part of the incoming light will be filtered out, while the red and the green part (higher wavelength) of the visible spectra is reflected, the additive effect of the red and the green spectra makes the corresponding body appear yellow (**Figure 1.3**).

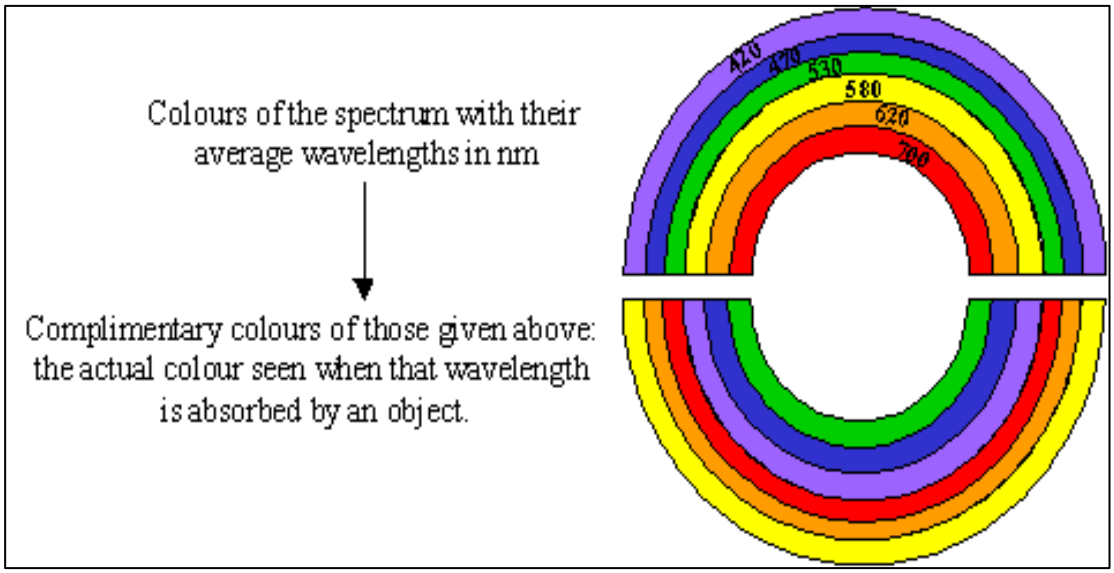



**Figure 1.3 Chromatic colors forming the visible part of spectrum and their complimentary colors as seen by the human eye (picture courtesy www.colormatters.com)**

## 1.2 Chemical Colors

Colors can result from physical (reflection or refraction as in case of rainbow formation), chemical (Organic dyes and pigments) and physico-chemical (e.g. gemstones) phenomena (4). Since chemical based colors forms the basis of this thesis, an insight into color chemistry forms the topic of this section.

**What gives the biological molecule a colour?**

The colour of a biological molecule, whether it is a case of chemical, physical or perceptual matters, is dependent on structure, the electron's actual state of energy, the size, solubility and elemental composition. Most biological pigments are large molecules, and contain either N or O, often both (5).

Electrons will, as a starting point, be in their resting state. When a molecule gets exposed to light of sufficient energy, the electrons will be raised to an excited state. When the electrons return to their resting states, energy will be released in a less energetic form (heat, light at shorter wavelengths). Almost all of the electronic orbitals participating in colouring of compounds are from d, p and n orbitals. Excited orbitals are denoted as d* and p*.

Transitions from d to d* requires more energy than provided by the visible wavelengths of light, that is why these compounds are sensed as colourless. The transitions from p to p* are less demanding in energy than d to d*, but the energy provided is beyond the visible wavelengths of light. Transition from n to d* may appear in the visible range of the spectra as pale yellow compounds, but the transition from n to p* requires the least energy, and can therefore happen at the visible wavelengths of light. The compounds excited from n to p* will appear red, blue or green.

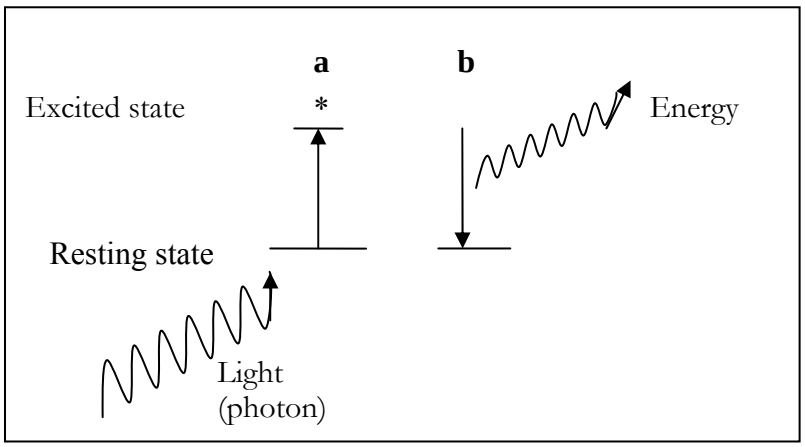



**Figure 1.4 a) An electron absorbs light so it rises to an excited state. b) The electron returns to the resting state and energy is released.**

## 1.3 Classification of colorants

For consumers the colorants may appear to be either natural or synthetic, but it is definitely more than that and is worthwhile to take a look at it.

Based on chemistry, colorants can be divided into organic or inorganic both of which are mostly synthesized chemically in the lab. The simple examples of which are carotenoids and titanium dioxide respectively. Most of the naturally occurring colorants that are currently in use are typically organic colorants. Furthermore, organic colorants can be classified as pigments and dyes based on their solubility in the media; if they are insoluble and require a fixative to adhere to the surface of the substrates, they are termed as pigments while the dyes are almost soluble in the media to which they are added and do not require any fixatives. Thus, carotenoids are dyes in oil but pigments in water. However, the term "pigment" will be used throughout the thesis for colored substances in general for the uniformity.

As per the origin and legislation is concerned, food colorants can be categorized into: 1) natural 2) nature-identical and 3) synthetic. Natural colorants are pigments synthesized, accumulated or excreted by living organisms. They also include colorants made by the modification of materials from living organisms, such as caramel, vegetable carbon, and copper-chlorophyllin. Ironically, they are (except for carbon) not found in nature. Nature-identical colorants are chemically synthesized man-made pigments which are also found in nature. Examples include, ß-carotene, canthaxanthin, and riboflavin. Synthetic colorants are man-made colorants that are not found in nature; these are mostly azo-dyes. Consumers, however, perceive only natural and synthetic classes of colorants. Thus, it can be inferred that natural colors are regarded as safe and have a healthy image for the consumers.

### 1.3.1. Important chemical classes of natural food colorants

Although structurally quite diverse and derived from a variety of sources, natural food colorants can be grouped into five important classes (6); tetrapyrrole derivatives, isoprenoid derivatives, benzopyran derivatives, artefacts, and others (**Table 1.1**). Chlorophylls belong to the class tetrapyrrols which is a characteristic photosynthetic green plant pigment and is ubiquitous. Other members of photosynthetic apparatus include carotenoids that belong to the pigment class of isoprenoid derivatives. They impart characteristic yellow-orange-red color to many fruits. Anthocyanins are a group of

flavonoids that provide the red-purple shade to many fruits such as strawberries, elderberries, and black currants.

**Table 1.1 Classification of existing natural colorants** (6)

| Main Classes | Sub-classes / examples |
|---|---|
| Isoprenoid derivatives | Carotenoids / ß-carotene, lycopene |
| | Xanthophylls / |
| Tetrapyrrole derivatives | Chlorophylls / chlorophyll a |
| | Porphyrins / hemoglobin, myoglobin |
| | Bilins / Phycoerythrin, phycocyanin |
| Benzopyran derivatives | Anthocyanidins / Cyanidin, malvidin |
| | Flavonoids / |
| | Tannins / catechin, theaflavin |
| Artefacts | Melanins / eumelanin |
| | Melanoidins and caramels |
| Others | Anthraquinones / carminic acid |
| | Betalains / betanin |
| | Phenalones / curcumin |
| | Flavins / riboflavin |

They are an example of benzopyran derivatives. Melanins, melanoidins and caramels are grouped together since they are all complex polymeric molecules, with some similarity in structural units comprising the polymers. The distinction between the melanins and melanoidins and caramels is that melanins occur naturally and are responsible for many of the black, grey and brown colors found in plants and animals, Melanoidins and caramels, however, are formed by non-enzymatic browning reactions, usually during the heat processing of foods, melanoidins are produced as a result of Maillard reactions, essentially a reaction initiated between a reducing sugar and a primary or secondary amine, while caramelization reactions result from the effect of heat on sugars. The pigments of scale insects such as lac and cochineal belong to anthraquinone subclass. For example, carminic acid, which imparts orange-red-purple color shade; the color depends on the pH. Beet root is a major source of betalains which comprises of the red-purple betacyanidins and the yellow betaxanthins. Curcumin belongs to the subclass phenalones. The major source of which is the rhizome of the plant *Curcuma longa*. It imparts a characteristic greenish-yellow color. Another less widely used colorant that belongs to the subclass flavin is

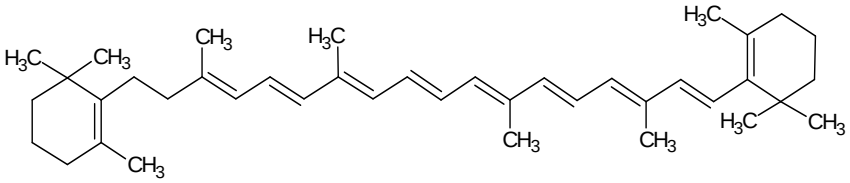
riboflavin. Structures representing different classes of natural colorants are shown in **Figure 1.5**.

## 1.4 Natural Vs Synthetic colorants: a quick preview

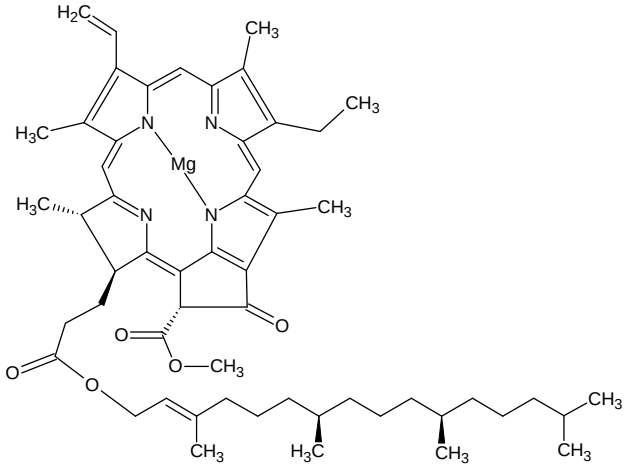
Use of dyes to color the food stuff have been used historically, however the detrimental effect of such food dyes became more prominent only after the invention of synthetic dyes. For example, it has been reported that synthetic colorant, fuchsine, was used as early as in 1860 in France as a wine additive (4), arsenic acid (toxic) was used as a reagent for its manufacture. Today, consumers are concerned about the eventual harmful effects of synthetic colorants; Fast Green dye has been shown to be an immunotoxic agent (7). Despite the fact that synthetic colorants are relatively stable, have higher color strength per mole of a compound, cheaper to produce, and are relatively more stable, natural colorants are preferred due to their safe and healthy image and the fact that they can be produced by eco-friendly means (4). The value of the international colorings market was estimated at around $ 1.15 billions in 2007 (€ 731 millions), up 2.5% from $1.07 billions (680 m) in 2004, according to Leatherhead Food International (LFI). Natural colorants now make up 31% of the colorant's market, compared with 40% for synthetics, according to LFI (8). Thus, it can be said that naturally derived colorants look set to overtake synthetic alternatives in market value due to the rising demand for the clean label ingredients.

**Figure 1.5. Some characteristic natural pigments representing different classes.**

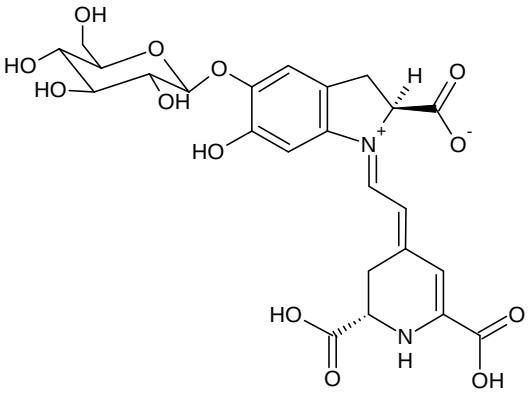
**A. ß-carotene (Isoprenoid derivative)**



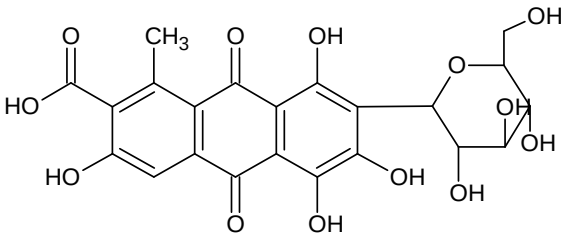

**B. Chlorophyll a (Tetrapyrrole derivative)**




**C. Betanin (Betalains)**




**D. Carminic acid (Anthraquinones)**



## 1.5 Legislative issues

In most countries, only certain dyes are permitted as food colors, and compound-specific purity standards are governed by strict regulation. The legislation specifies which colorants may be used; the sources of the colorants, the purity of the colorant, to which foods the colorant may be added, and at what level the colorant may be added to a specific food. The European Union (EU) directive, EU Directive 94/36/EC, often referred to as the Colors Directive, is implemented throughout the members state. Here the colors are listed together with conditions for their use. In the United States (US), the use of colors is outlined in Code of Federal Regulations, Title 21 (21CFR). A number of countries also follow the US legislation. The Australian and Japanese legislations on colors in food are also used as the basis for local regulation in a number of other Asian countries. Legislation is often influenced by local, traditional usage of colorants as in case of *Monascus* colorants (pigments of an ascomycetous fungus *Monascus* used as fermentatively produced red rice powder), lac, and gardenia based colorants. None of these are permitted to be used in the EU and the US, partly due to the fact that their sources are too alien for these societies.

There are many similarities between the EU and US legislation about the colorants that are allowed (**Table 1.2**), however, there are striking differences when it comes to the sources of the colorants and the foods in which the colorants can be applied. For example, in the USA, sodium copper chlorophyllin may only be made from alfalfa (*Medicago sativa*) and only be used in citrus-based dry beverage mixes (9), whereas in the EU, allowed sources are alfalfa, grass, nettle, and edible plant material (1), and a wide range of foods may be colored.

### 1.5.1. Quality control issues

The extent of quality control depends on the type of color product, the information available on the certificate of analysis, the expected processing conditions, the finished product and the standard quality control procedures of the food or beverage manufacturer. In some cases the color strength or color hue and intensity may be the most important parameter. In others the microbiological standard and absence of pathogens is also important. For powder formulations to be used in dry blends, the particle size is relevant, whereas density can be a key point if the color is volumetrically measured by e.g. flow meter during the production process (10).

**Table 1.2** Allowed natural and nature-identical, and synthetic colorants in the EU and the US (1, 11)

| EU name | E-number | US name | US. CFR number |
|---|---|---|---|
| Curcumin | E 100 | Turmeric | 73.600, 73.615 |
| Riboflavin | E 101 | Riboflavin | 73.450 |
| Cochineal, carminic acid, carmines | E 120 | Cochineal extract, carmine | 73.100 |
| Chlorophyll(in)s | E 140 | Not allowed | |
| Copper complexes of Chlorophyll(in)s | E 141 | Sodium copper chlorophyllin | 73.125 |
| Caramel | E 150 | Caramel | 73.85 |
| Vegetable carbon | E 153 | Not allowed | |
| Mixed carotenes | E 160a (i) | Carrot oil | 73.300 |
| ß-carotene | E 160a (ii) | ß-carotene | 73.95 |
| Annatto, bixin, norbixin | E 160 b | Annatto extract | 73.30 |
| Paprika extract | E 160c | Paprika | 73.340, 73.345 |
| Lycopene | E 160d | Not allowed | |
| ß-Apo-8'-carotenal | E 160e | ß-Apo-8'-carotenal | 73.90 |
| Ethyl ester of ß-apo-8'-carotenoic acid | E 160f | Not allowed | |
| Lutein | E 161b | Not allowed | |
| Canthaxanthin | E 161g | Canthaxanthin | 73.75 |
| Beetroot red | E 162 | Dehydrated beets | 73.40 |
| Anthocyanins | E 163 | Grape color/skin extract | 73.169, 73.170 |
| | Not allowed | Cottonseed flour | 73.140 |
| | * | Fruit juice | 73.250 |
| | * | Vegetable juice | 73.260 |
| | * | Saffron | 73.500 |
| Synthetic | | | |
| Tartrazine[1] | E 102 | F,D & C Yellow No. 5 | |
| Quinoline Yellow | E 104 | D & C Yellow No. 1C | |
| Sunset Yellow FCF[2] | E 110 | F,D & C Yellow No. 6 | |
| Carmoisine | E 122 | Ext D & C Red No. 10 | |
| Amaranth | E 123 | | |
| Ponceau 4R | E 124 | F,D & C Red No. 2 | |
| Erythrosine | E 127 | F,D & C Red No. 3 | |
| Red 2G | E 128 | Ext D & C Red No. 11 | |

| | | |
|---|---|---|
| Allura Red AC[3] | Banned | F,D & C Red No. 40 |
| Patent Blue V | E 131 | |
| Indigo Carmine[4] | E 132 | F,D & C Blue No. 2 |
| Brilliant Blue FCF[5] | E 133 | F,D & C Blue No. 1 |
| (Food) Green S | E 142 | |
| Black PN | E 151 | |
| Brown FK | E 154 | |
| Chocolate brown HT | E 155 | |

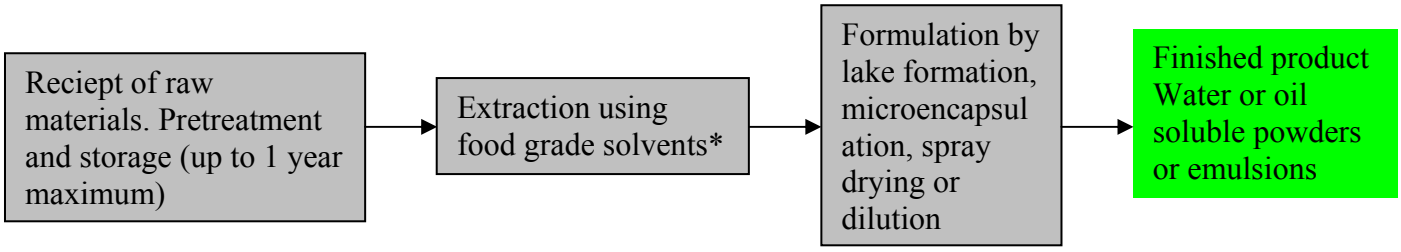
*Allowed as a food ingredient (not an additive) that does not require an E-number, [1]Known to cause thyroid and lymphatic tumors (12), [2]Known to cause kidney tumors, chromosomal damage and allergy (12), [3]Known to cause lymphatic tumors and hyperactivity (12), [4]Known to cause brain tumor (12), [5]Known to cause chromosomal damage (12).

## 1.6 Natural food colorants currently in use

### 1.6.1. Production of natural food colorants

Existing natural food colorants are derived from traditional sources that include mostly flowering plants. Other sources include scale insects such as cochineal and lac, and microorganisms such as filamentous fungi and cyanobacteria. Typically, they are extracted by using food grade solvents, and sold in the form of either dried powders or formulated into some kind of liquid forms. They may be stabilized for instance by encapsulation based on their application in water or oil based systems. A schematic presentation of the key steps involved in the production of natural colorants is depicted in **Figure 1.6** and the key steps are discussed briefly as follows:

**Extraction**. Lipid-soluble pigments such as chlorophyll and carotenoids are usually extracted with organic solvents, which are subsequently removed, yielding an oleoresin rich in pigments, but also containing other material such as triglycerides, sterols, wax, and other lipid-soluble compounds. Water soluble pigments such as carminic acid and anthocyanins are generally extracted with water or lower alcohols (1).




* Those allowed in the EU are water, ethyl acetate, acetone, n-butanol, methanol, ethanol and hexane, while those allowed in the US are isopropanol, methanol, ethanol, hexane, and acetone.

**Figure 1.6. Generic figure showing steps involved in the industrial production of natural colorants.**

**Formulation**. Formulation is the process in which the extracted colorant is mixed with other components to serve a number of purposes, For instance, to get a new color shade by mixing two or more colorants or to increase the range of application as in case of lipid soluble carotenoids by emulsification so that it may be used in the water based systems (1).

Lake formation is one of the ways of formulating colorants. It is the process of precipitating soluble pigments with aluminium cations in the presence or absence of calcium cations. lakes are soluble at low and high pH. Lake formation confers a high degree of stabilization.

Increasing stability is also an important reason for formulating colorants. Antioxidants such as α-tocopherol and ascorbic acid are added to the pigment formulations to inhibit color fading. The pigments may also be microencapsulated with hydrocolloids such as gelatin, pectin etc. which, besides making oil-soluble colors dispersible in water, create a physical barrier around the pigment, protecting it from degradation.

Formulation of colorants may also be done to enhance handling of them. Thus, many oleoresins are highly viscous liquids or semi-solids, but by diluting them with vegetable oil a more easy-to-handle colorant of lower strength is obtained. In some applications, a powder is preferred over a liquid. It is possible to spray-dry many colorants using maltodextrin as a carrier to get a powder that can easily be dissolved or dispersed in water (1).

### 1.6.3. Merits Vs Demerits of natural food colorants currently in use

Since natural colorants are extracted from natural sources, they are in most cases mixtures of varying composition and, therefore, not easy to characterize with respect to purity and contaminants. Anthocyanins and flavonoids form one such an example that is derived from many fruits and vegetable sources. Their use in a pure form as food additives is limited mainly for three reasons:

1) They are expensive to isolate in pure form and large quantities from natural sources.

2) They are difficult to synthesize.

3) The color is often pH dependent as in case of anthocyanins. An increase of pH enhances blue hues, whereas, under acidic conditions, an orange to red color is observed (13).

The issue of stability of pigments is not to be overlooked. Pigments are highly unsaturated compounds and are prone to light, heat, and oxygen disintegration. However, there exists a large difference in the stability of different classes of existing natural food colorants. Vegetable carbon and caramel are very stable towards heat, light, and oxygen. Carminic acid and carmine are also quite stable but not as stable as vegetable carbon and caramel. On the other hand, turmeric is rapidly bleached by light and beet root pigments turn brownish even under mild heating conditions (1).

Carotenoids such as ß-carotene (**Figure 1.5 A**) has some nutritional value as an additive in margarine, since it is metabolized to retinol (Vitamin A) in the human body.

### 1.6.4. What is food colorant industry looking for?

From the foregoing discussion on the state-of-the-art of the existing natural food colorants it can be said that the future prospects of natural food colorant industries lie in the three key areas *viz*. robust production of colorants, discovery of new sources with existing or known colorants and improved functionality. Therefore, food colorant industry is looking for:

- Safe and healthy colorants.
- Constant supply of raw materials independent of seasonal changes.
- Cheaper and reliable production.
- New colorants with better functionality.

## 1.7 Production of natural food colorants using biotechnological tools and sources: State-of-the-art

Use of biotechnology (from tools to sources) would offer solutions to the problems, the food colorant industry is facing today. Fermentative production of colorants has a number of advantages that include: cheaper production, higher yields, readily available raw materials, and no seasonal variations.

Among the sources of natural food colorants, use of plant cell cultures in bioreactors, for instance, anthocyanins from carrot (*Daucus carota*), seem to provide one possible solution in terms of production (14). However, the lower yield could be an obstacle in such a means of production. Genetic manipulation of existing plant sources could lead to major improvement, however the naturalness of the colorant would be lost (at least partially). Moreover, it is not allowed in the EU at the moment.

Among microbes, microalgae such as *Spirulina* (phycocyanin), *Haematococcus* (astaxanthin), and *Dunaliella* (ß-carotene and other carotenoids) are already explored as sources of natural food colorants (14) though the lower productivity and contamination in the open culture system, where they are grown, could be a major bottleneck (15). Recently, a blue-green polyphenolic antioxidant pigment called marennine from the Diatom *Haslea ostrearia* has been reported (16). This may serve as a promising food coloring additive.

Bacterial sources of food colorants such as zeaxanthin by *Flavobacterium*, canthaxanthin (mostly used in aqua feed) from the photosynthetic bacterium *Bradyrhizobium* spp., and *Halobacterium* spp. have been investigated. Astaxanthin production by *Agrobacterium aurantiacum, and Paracoccus carotinifaciens* have been investigated. Case of aryl-carotenoids such as isorenieratene and hydroxyl derivatives production by *Brevibacterium aurantiacum*; a bacterium associated with red-smear ripened soft cheeses, forms an interesting avenue to be explored further. This is because the pigments produced by this bacterium, thus, have been consumed for a long time. Apart from carotenoids, rubrolone is known to be produced by *Streptomyces echinoruber* (17).

Among yeasts, the commercial success has been with the case of astaxanthin production by *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*). A major drawback of this production process is that disruption of the cell wall of yeast biomass is required prior to its use as food additive. Another example of production of carotenoids is by the yeast *Rhodotorula* spp., *egs*., *R. glutinis, R. gracilis, R. rubra, and R. graminis.* The main

carotenoids produced by these yeasts are torulene and torularhodin, with minute quantity of ß-carotene (17).

### 1.7.1. Fungi as sources of food colorants

Fungi, particularly ascomycetous and basidiomycetous (mushrooms) fungi, and lichens (symbiotic association of a fungus with a photosynthetic partner usually either a green alga or cyanobacterium) are known to produce extraordinary range of colors that include several chemical classes of pigments. Only 355 species from the fungal kingdom have been explored for pigments and colorants, out of those 37 belonged to lichens and 318 belonged to mushrooms and other fungi (18). Mushrooms and lichens have a rich history as sources of pigments for textile coloring. Mycelial extracts of some promising mushrooms such as *Chroogomplus vinicolor* gives red tints, *Bankera violascens* gives greens and *Collybia iocephala* gives blues. They have a tremendous potential for dying wool and silk fabrics (18). However, such fungi are difficult to grow under lab conditions and therefore are not suitable for large scale industrial productions.

On the other hand ascomycetous fungi are more suitable for biotechnological production because they can be grown in a relatively easier way to give high yields using existing culture techniques. Food colorants from ascomycetous fungi have been explored with few successful attempts. Carotenoids such as ß-carotene and lycopene have been known to be produced by fungal cell factories. The successful industrial production of ß-carotene by *Blakeslea trispora* is the best example to be given. DSM (The Netherlands) was the first company to produce ß-carotene from this fermentative source. Today, there are two other industrial productions of *B. trispora* fungal ß-carotene, the first in Russia and Ukraine, the second in Leon (Spain). Another explored fungal sources of ß-carotene are *Mucor circinelloides* (zygomycete fungus), and *Phycomyces blakesleeanus*. Widely known vitamin, riboflavin (vitamin B2) is a yellow food colorant that is fermentatively produced by the fungi *Eremothecium ashbyii* and *Ashbya gossypi* (17). As far as polyketide class of fungal pigments is concerned, species of the ascomycetous fungus *Monascus* have been utilized for making Oriental foods, especially in Southern China, Japan and Southeastern Asia, such as red rice wine, red soya bean cheese and anka (red rice). Anka was traditionally produced by inoculating moistened rice with *Monascus*, and the product contained various pigments linked to proteins, peptides, and amino acids. Annual consumption of *Monascus* pigments in Japan alone moved from 100 tonnes in 1981 to 600 tonnes at the end of the nineties and was valued at $1.5 million. New food applications,

like the coloration of processed meats (sausage, ham), marine products like fish paste, surimi and tomato ketchup were described (17).

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# *Chapter 2*

# *Introduction*

## 2.1. Fungal Pigments: Secondary metabolites of known or unknown function

Fungal pigments belong to microbial secondary metabolites and therefore play no obvious function in cell growth or development. Secondary metabolites are synthesized by cells that are no longer undergoing balanced growth. Secondary metabolism, thus, involves mainly synthetic processes and is species-, often strain-specific. On the other hand, primary metabolism includes all the anabolic and catabolic processes that are finely balanced to keep the organism alive. It is essentially an identical process for all living organisms. Secondary metabolites are produced from a few key intermediates of primary metabolism, when a substrate other than carbon becomes limited. Thus, according to Bu'Lock (1), it is the process of secondary metabolism which is important for the organism rather than the secondary metabolites themselves. In other words, secondary metabolism provides a route for the removal of intermediates which would otherwise accumulate and thus enables the primary processes leading to these intermediates to remain operational during the time of stress.

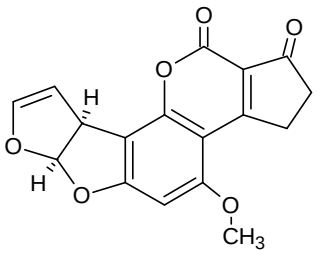
Although secondary metabolites do not have any obvious function in cell growth, it has been suggested that certain secondary metabolites may have a metabolic role, such as mediating passage of certain molecules across the plasma membrane (valinomycin, alamethicin), and playing a role in competition or combating other microbes in natural micro-environments (2). Secondary metabolites have often attracted commercial interest because of their biological effect on other organisms. The sources of secondary metabolites are plants and microbes. Among microbes, fungi provide a vast array of secondary metabolites that often have an ecological role in regulating the interactions between plants, microorganisms, insects and animals. Pigments are no exception. Like plant pigments, fungal pigments also play ecological role for the organism that produces them. For example, melanin; also known to be produced by bacteria, (polymerized phenolic and/or indolic compounds) provides resistance to a variety of adverse environmental factors such as desiccation and irradiation (3). If the producer is in soil, then melanins accumulate in the soil humus, where they may play an important role in soil chemistries (4). Carotenoids are known to have a protective action against photooxidation. Flavins act as cofactors in enzyme catalysis. However, the specific role of all kinds of pigments, for the organisms which produce them, like many other secondary metabolites is scarcely found in the literature and yet unknown.

## 2.2. Biosynthesis of polyketide class of secondary metabolites and pigments

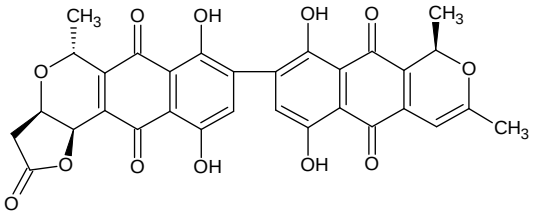
The classes of secondary metabolites can be represented as (5):

- Polyketides and fatty acids
- Terpenoids and steroids
- Phenylpropanoids
- Alkaloids
- Specialized amino acids and peptides
- Specialized carbohydrates

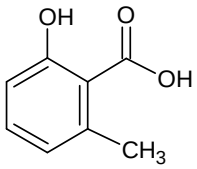
In this section the focus will be on polyketide class of secondary metabolites. Polyketides represent a major group of structurally and functionally diverse secondary metabolites (**Figure 2.1**). Polyketides are synthesized by the catalytic action of the multifunction enzyme polyketide synthases (PKSs). Most PKSs share a minimum of four different domains, *viz*. ketosynthase (KS), acetyltransferase (AT), ketoreductase (KR), and acyl carrier protein (ACP). In addition, the domains dehydratase (DH), thioesterase (TE), and cyclase (CYC) are also found in PKSs. PKSs have been classified into three categories based on their enzyme architecture and gene organization. The three types of PKSs are; type I) bacterial modular PKS e.g. erythromycin PKS, rapamycin PKS, and iterative fungal Pks e.g. 6-Methylsalicylic acid (MSA) PKS, lovastatin PKS, and type II) bacterial aromatic PKS e.g. tetracycline PKS, actinorhodin PKS, and type III) plant PKS. However, recently it was suggested to move away from the above-mentioned classification of PKSs since there are some PKSs in the transitional stages of the different type (6). Polyketides are formed by the condensation of an acetyl unit, or other acyl unit with malonyl units, with simultaneous decarboxylation as in fatty acid biosynthesis but without obligatory reduction of the intermediate ß-dicarbonyl system. This results in the biosynthesis of a carbon chain, with alternate carbon atoms coming from the methyl and carboxyl groups of the acyl building block. The acetate origin of these compounds leads to a formation of even-numbered carbon chains. The polycarbonyl compounds hence formed serve the substrates for various cyclases that lead to aromatic compounds; representing typical fungal metabolites (5).



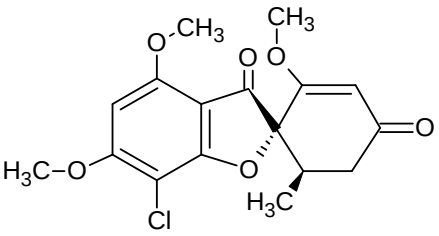
Aflatoxin A1-fungal
(mycotoxin)



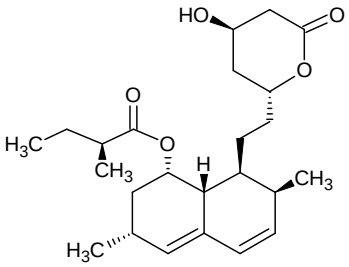
a-actinorhodin-bacterial
(antibiotic)



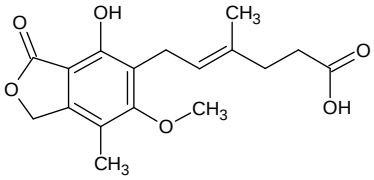
6-methylsalicylic
acid (antibiotic)



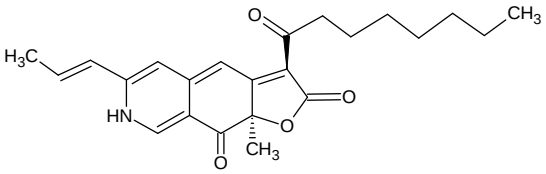
Griseofulvin-bacterial
(antifungal)



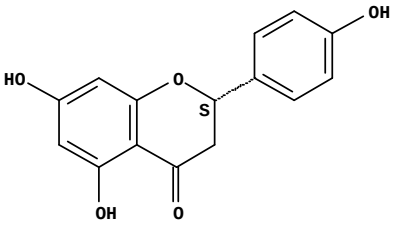
Lovastatin-fungal
(Cholesterol lowering agent)



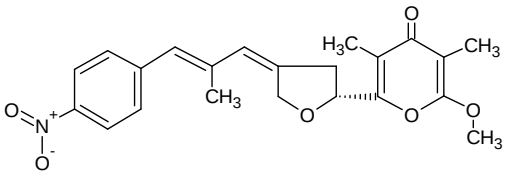
Mycophenolic acid-fungal
(immunosuppressant)



Monascorubramine-fungal
(food colorant)



Naringenine-plant based
(antioxidant)



Aureothin-bacterial
(antitumor)

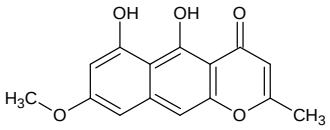
**Figure 2.1 Some exemplary polyketides representing structural and functional diversity**

Polyketides can be classified on the basis of their biosynthesis indicating the number of "$C_2$-units" that have contributed to the polyketide chain and according to the type of cyclization the precursor has undergone. The terms triketide, tetraketide, pentaketide, etc., denote compounds derived from three, four or five "$C_2$-units" respectively (7).

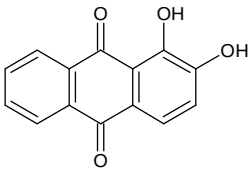
Fungal polyketide pigments represent tetraketides to octaketides and some of them involve mixed biosynthesis meaning that they involve other pathways (such as amino acid, or terpenoids) in addition to the polyketide pathway. They typically represent anthraquinones, hydroxyanthraquinones, naphthoquinones and azaphilone structures. **Figure 2.2** shows the extraordinary color range of polyketide pigments of fungi. The variation in the domains of iterative type I fungal PKSs could be one of the prime reasons for the unique structural and chemical diversity found in the polyketie pigments resulting in an extraordinary range of colors. They contain from none to 3 reductive domains, some contain a methyltransferase (MT) domain, and some the thioesterase (TE) domain and finally some PKSs contain tandem acylcarrier protein (ACP) motifs for unknown reasons. Thus, it can be said that the complexity involved in the biosynthesis of polyketide pigments could be due to the involvement of varied polyketide sunthases forming a multistep biosynthetic pathways. Due to the rudimentary knowledge about fungal polyketides, they have not been studied and heterologously expressed in other hosts at the same level as bacterial polyketides.

### 2.2.1. Role of fungal polyketide pigments

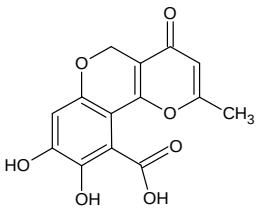
Like the other classes of fungal pigments, role of polyketide pigments is still not specifically known but the following information would give a fair idea about the diverse roles they could play for their producers. Many of the polyketide pigments have an antibiotic activity; therefore, their production confers upon the organism an advantage in its natural environment. For example, red pigment bostricoidin produced by *Fusarium oxysporum* (formerly *bostrycoides*) (8). Fungal polyketide pigments may be synthesized as the intermediates during an important secondary metabolite, *e.g*. mycotoxin, biosynthesis or as intermediates for unutilized branch pathway products. For example, anthraquinone pigments such as norsolorinic acid, averantin, averufin, versicolorin C, versicolorin A, versicolorin A hemiacetal and nidurufin, have been shown to be on or associated with the aflatoxin biosynthetic pathway in Aspergillus spp., for instance, *Aspergillus flavus* (9).



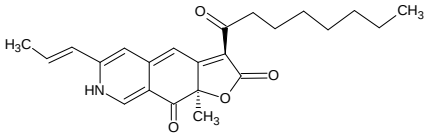
Rubrofusarin **(red)**



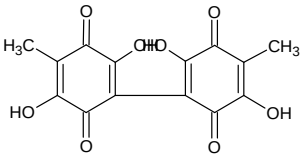
Alizarin **(orange)**



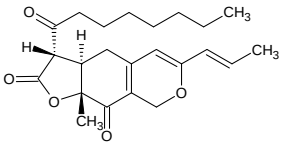
Citromycetin **(yellow)**



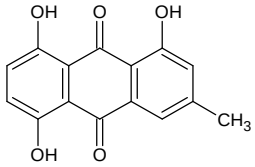
Monascorubramine **(purple-red)**



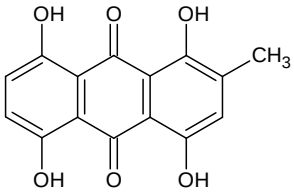
Oosporein **(purple-yellow)**



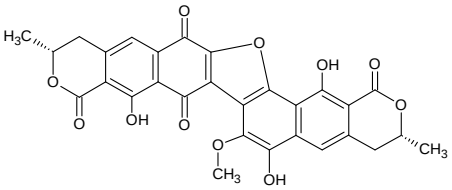
Ankaflavin **(yellow)**



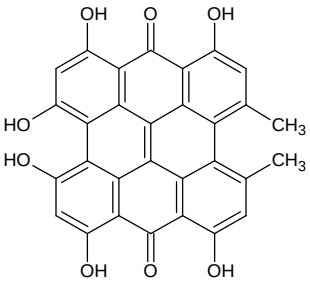
Helminthosporin **(brown)**



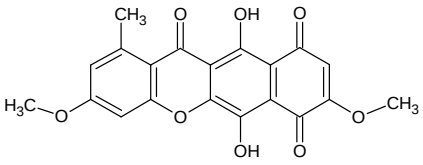
Cynodontin **(bronze)**



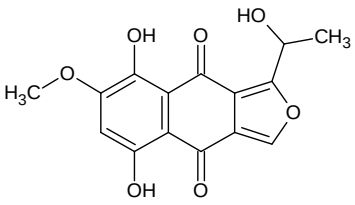
Viopurpurin **(blue)**



Hypericin **(purple)**



Bikaverin **(dark red)**



Nectriafurone **(yellow-brown)**

**Figure 2.2 Some exemplary fungal polyketide pigments depicting the extraordinary color range.**

Thus, it can be inferred that these pigments may be held in storage for subsequent rapid conversion to aflatoxin and to do that, environmental factors such as culture conditions and media composition (under lab conditions) or the harsh environment (in their natural habitat) constituted by a diverse array of competing organisms, could be decisive. Another possible explanation of their accumulation could be that they are formed as excessive intermediates may be needed to drive an unfavourable biosynthetic step(s) by mass action.

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## 2.3. Review

The following review article was written at the onset of my PhD study, thus, the two introductory chapters were written based on the latest references available.

**Exploring fungal biodiversity for the production of water-soluble pigments as potential natural food colorants.**

Sameer AS Mapari, Kristian F Nielsen, Thomas O Larsen, Jens C Frisvad, Anne S Meyer, and Ulf Thrane.

Current Opinion in Biotechnology 2005, **16**: 231-238, Copyright [2005] ELSEVIER.

# Exploring fungal biodiversity for the production of water-soluble pigments as potential natural food colorants

Sameer AS Mapari[1], Kristian F Nielsen[1], Thomas O Larsen[1], Jens C Frisvad[1], Anne S Meyer[2] and Ulf Thrane[1]

The production of many currently authorized natural food colorants has a number of disadvantages, including a dependence on the supply of raw materials and variations in pigment extraction. Fungi provide a readily available alternative source of naturally derived food colorants that could easily be produced in high yields. The recent authorization of a fungal food colorant has fuelled research to explore the extraordinary chemical diversity and biodiversity of fungi for the biotechnological production of pigments as natural food colorants. These studies require an appropriate use of chemotaxonomic tools and *a priori* knowledge of fungal metabolites to carry out intelligent screening for known or novel colorants as lead compounds. Such screening would result in the preselection of some potential pigment producers and the deselection of pathogenic strains and toxin producers. With advances in gene technology, in the future it should be possible to employ metabolic engineering to create microbial cell factories for the production of food colorants.

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## Introduction

The use of food colorants as additives in the food industry is a significant factor for both food manufacturers and consumers in determining the acceptability of processed food [1]. Currently, the European Union has authorized approximately 43 colorants as food additives, whereas approximately 30 colour additives are approved for use in the United States [2,3]. The legislation does not distinguish between synthetic and natural colour additives; however, in both Europe and the US several of the listed colour additives are derived from natural sources (Table 1) by physical and/or chemical extraction. The current commercial, naturally derived food colorants have several drawbacks (Table 1). Anthocyanins are flavonoids and are characterized by their basic flavylium cation structure (Figure 1a). The violet, purple colours of anthocyanins are sensitive to oxidation, bleaching by sulfur dioxide and vary with pH, limiting their application to acidic foods and beverages. Also, betanins, carotenoids and chlorophyll pigments contain labile hydrogens (Figure 1a) and are easily decolourized by oxidation, making them sensitive to light, heat and oxygen. These features limit the robustness of these colour additives during the processing, storage and display of the foods to which they have been added (Table 1). The food colorant curcumin has a different problem — curcumin is the major pigment of turmeric and carries a spicy, curry flavour, which limits its use as a food colorant. Furthermore, the fact that the current, naturally derived colorants are extracted from sources such as fruit skins, seeds or roots (see Table 1) means that the colour manufacturers are usually dependent on the availability and external supply of raw materials for the colour extraction. Obviously, the pigment profile in the natural sources is prone to variation and the extracted pigment profile is influenced by the extraction procedure employed. Thus, the chemical composition, including the presence of minor components, and the colour and stability properties of the current, commercial naturally derived colour additives vary significantly among different suppliers and from batch to batch [4]. To say the least, many consumers are likely to be unaware of the exotic sources of some of the currently authorized so-called natural colorants. The red colorant carmine (Figure 1b), for instance, is an extract of the female cochineal insect (Table 1) and to produce 100 g carmine colorant approximately 14 000 insects are required [5]. Many of the currently authorized 'natural' food colorants have been accepted by tradition. It has been claimed that if they were to be introduced into industrial food manufacturing today, natural colorants of similar variable quality and exotic origin might not be accepted [4,5]. This provides a large incentive to identify alternative ways in which natural colours can be produced in the future.

An alternative route for the production of natural food colorants is through the application of biotechnological tools to microorganisms. Microalgae and several classes of fungi are known to produce a wide range of excreted

**Table 1**

**Authorised naturally derived food colorants and their major sources.**

| Pigments | Sources | Colour range | EU code | Comments |
|---|---|---|---|---|
| Anthocyanins | Elderberries, black grape skin, black carrots, red cabbage | Pink/red to mauve/blue depending on pH | E163 | Colour is pH-dependent, heat-sensitive and subject to oxidation |
| Betanin | Red beetroots | Pink to red | E162 | Heat, light and oxygen-sensitive |
| Caramel colour | Food-grade carbohydrates | Brown | E150 (A–D) | Commonly used colouring agent |
| *Carbo medicinalis* | Plant | Black | E153 | From burnt plant material; banned in USA |
| Carminic acid and carmine | Female cochineal insect from Peru and Equador | Orange to red<br>Pink to red | E120 | Price-sensitive |
| Carotenoids | | | | |
| β-carotene[a] | Palm oil | Yellow to orange | E160a | Provitamin A activity makes carotenoids favourable from both a nutritional and an aesthetic point of view. They exhibit good pH stability in most foods, are sparingly oil soluble, easily oxidised and have a limited colour range |
| bixin or norbixin | Seeds from Annatto (*Bixa orellana)* from South America | Orange | E160b | |
| capsanthin/capsorubin | Paprika (*Capsicum annum L.*) | Reddish orange | E160c | |
| lycopene | Tomato (*Lycopersicum esculentum*) | Orangish red | E160d | |
| lutein | Marigold (*Tagetas erecta)* | Golden yellow | E161b | |
| canthaxanthin | Salmon, shrimp and flamingos | Orangish pink | E161g | |
| Chlorophyll | Grass, lucerne and nettle | Green to olive | E140 | Subject to photo-oxidation |
| Chlorophyllins (copper complex of chlorophyll) | Grass, lucerne and nettle | Bluish green | E141 | Not usually claimed as 'natural' on food labels |
| Curcumin | Plant rhizome *(Curcuma longa)* from India | Orange-yellow | E100 | Must be debittered to avoid its odour and sharp taste |
| Riboflavin[a] | Semisynthetically using ribose produced by bacterial fermentation | Yellow | E101 | Green fluorescence, light-sensitive and bitter taste |

[a] β-Carotene and riboflavin are also fermentatively produced by the fungi *Blakeslea trispora* and *Ashbya gossypi*, respectively.

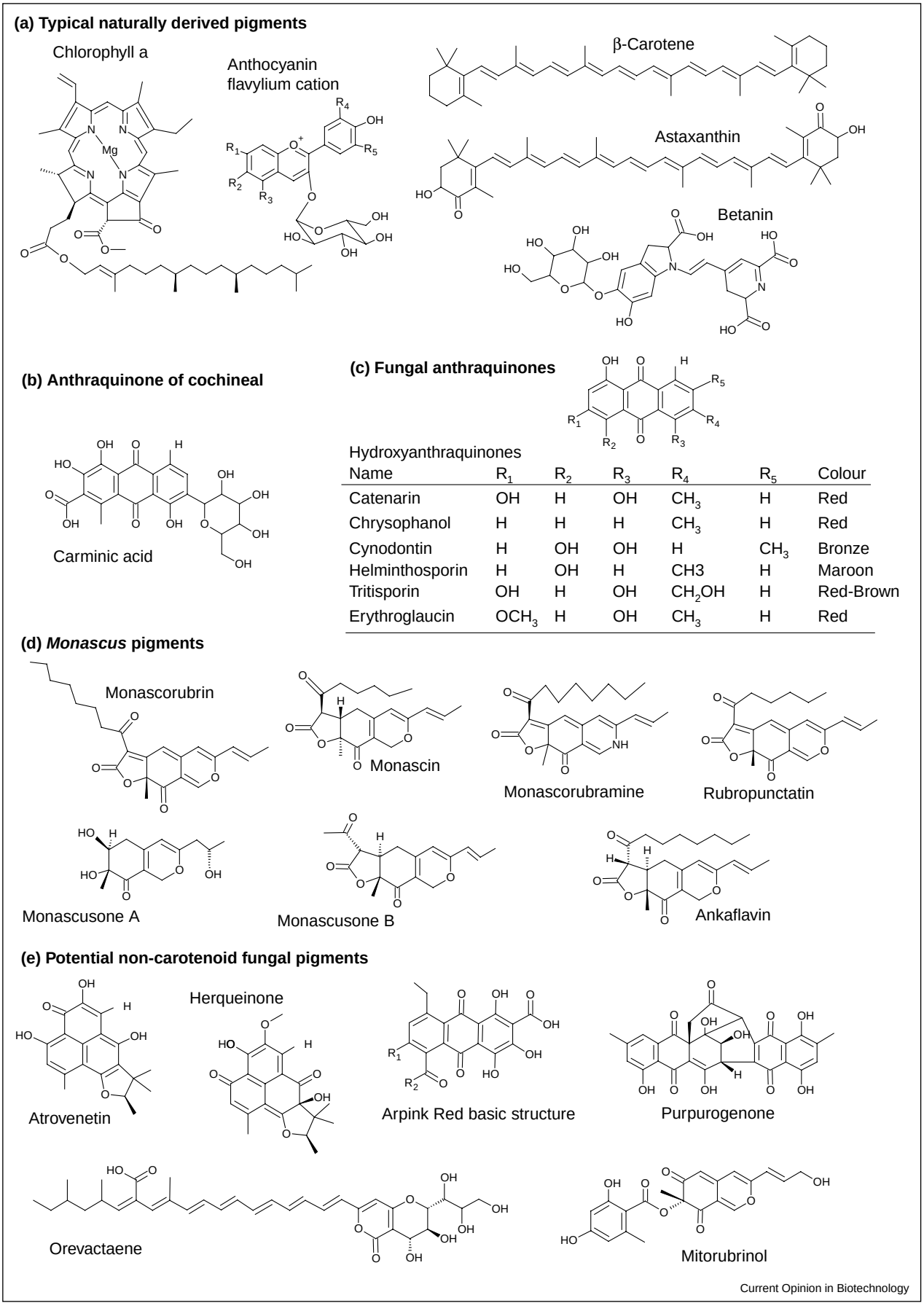
water-soluble pigments, but the low productivity of algal cultures is a significant bottle neck for their commercialization [6]. Pigments of basidiomycetous fungi have been used in the past for dying wool and silk [7], but such fungi are difficult to grow under laboratory and industrial large-scale conditions. Our attention is now drawn to the ascomycetous fungi. The use of such fungi to colour foodstuffs is not a novel practice, the use of *Monascus* pigments in food has been carried out traditionally in the Orient for hundreds of years [8]. Considering the apparent heat and pH stability of the *Monascus* derivatives during food processing, taken together with the socio-climatic independence of such a readily available raw material, fungi seem to be well worth further exploration as an alternative source of natural colorants. The appropriate use of fermentation physiology together with metabolic engineering [9] could allow the efficient mass production of colorants from fungi. With recent advances in gene technology, attempts have been made to create cell factories for the production of food colorants through the heterologous expression of biosynthetic pathways from either already known or novel pigment producers [10•,11].

Until recently, pigments from fungi were mainly used as a taxonomic tool for identification and species differentiation and were not studied as potential natural food colorants [12]. Furthermore, much of the earlier work on producing colorants from fungi focused on carotenoid pigments, the production of which has already been commercialized [13•]. In this review, we focus instead on fungi that produce water-soluble non-carotenoid pigments that are similar in structure and functionality to those derived from traditional sources. We consider how fungi can be screened for the production of either existing or novel colorants, which can then be used as leads for developing compounds with improved functionality in food systems. We discuss fungal biodiversity and the application of chemotaxonomy to carry out intelligent screens for such pigments with an aim of biotechnological production.

## Biodiversity of filamentous fungi: a promising source of colorants

Like plants, filamentous fungi synthesize natural products because they have an ecological function and are of value to the producer [14]. Depending on the type of compound, they serve different functions — varying from a protective action against lethal photo-oxidations (carotenoids) to protection against environmental stress (melanins) and acting as cofactors in enzyme catalysis (flavins).

**Figure 1**


(a) Typical naturally derived pigments
Chlorophyll a
Anthocyanin flavylium cation
β-Carotene
Astaxanthin
Betanin
(b) Anthraquinone of cochineal
Carminic acid
(c) Fungal anthraquinones
Hydroxyanthraquinones
Name R1 R2 R3 R4 R5 Colour
Catenarin OH H OH CH3 H Red
Chrysophanol H H H CH3 H Red
Cynodontin H OH OH H CH3 Bronze
Helminthosporin H OH H CH3 H Maroon
Tritisporin OH H OH CH2OH H Red-Brown
Erythroglaucin OCH3 H OH CH3 H Red
(d) Monascus pigments
Monascorubrin
Monascin
Monascorubramine
Rubropunctatin
Monascusone A
Monascusone B
Ankaflavin
(e) Potential non-carotenoid fungal pigments
Atrovenetin
Herqueinone
Arpink Red basic structure
Purpurogenone
Orevactaene
Mitorubrinol
Current Opinion in Biotechnology

Besides providing functional diversity to the host, these pigments exhibit a unique structural and chemical diversity with an extraordinary range of colours. Several characteristic non-carotenoid pigments are produced by filamentous fungi, including quinones such as anthraquinones and naphthaquinones [15,16], dihydroxy naphthalene melanin (a complex aggregate of polyketides) [17], and flavin compounds such as riboflavin [18••]. Anthraquinone (octaketide) pigments like catenarin, chrysophanol, cynodontin, helminthosporin, tritisporin and erythroglaucin (Figure 1c) are produced by *Eurotium* spp., *Fusarium* spp., *Curvularia lunata* and *Drechslera* spp. [18••]. Yellow pigments epurpurins A to C were isolated from *Emericella purpurea* [19] and azaphilone derivatives (hexaketides), falconensins A–H and falconensones A1 and B2, are produced both by *Emericella falconensis* and *Emericella fructiculosa* [20].

*Monascus* spp. are known to produce well-known azaphilone pigments like monascorubrin, rubropunctatin [21] and, more recently, monascusones from a yellow *Monascus* mutant have been identified (Figure 1d) [22]. Monascorubrin and rubropunctatin have a unique structure responsible for their high-affinity for compounds with primary amino groups (so-called aminophiles). Reactions with amino acids yield the water-soluble red pigments, monascorubramine and rubropunctamine. In fact, various pigment derivatives with improved functional properties in the colour range of orange-red to violet-red can be produced by *Monascus* fermentations in the presence of different amino acids [23]. However, *Monascus*-fermented rice has been found to contain the mycotoxin citrinin [24]; the production of citrinin together with pigments clearly limits the use of *Monascus* as a producer of natural food colorants. In a recent review article, however, it was suggested that citrinin is only produced by some and not all *Monascus* strains [25].

A new natural food colorant of fungal origin called Arpink Red™ is manufactured by ASCOLOR BIOTECH in the Czech Republic (http://www.ascolor-biotec.cz). The red colorant is an extracellular metabolite of the anthraquinone class and is claimed to be produced by a variety of *Penicillium oxalicum*. It is also said to confer anticancer effects when used in food supplements [26,27]. Some other promising fungal pigments of commercial importance are listed in Table 2 together with their sources. They exhibit a broader colour range when compared with the limited colour range of carotenoids and, as these pigments are water-soluble, they do not require chemical modification or the use of carriers and stabilizers for dispersion in foods. The chemical diversity of these pigments is illustrated in Figure 1c,d,e and their structural similarity to some of the existing colorants of plant or animal origin is evident. For example, fungal anthraquinones are similar to carminic acid (Figure 1b) and the basic structure of Arpink Red™ (Figure 1e) is reported to be related to the carmines or kermes red colorant group [28]. It is apparent that there is significant scope to discover further food colorants from fungi, these compounds could be already known or novel lead compounds.

## Screening and identification of potential pigment-producing filamentous fungi

To the best of our knowledge, the molecular and genetic basis for the biosynthesis of any fungal pigment has not yet been examined in detail; thus, genomic screening for pigment producers is not feasible at this premature stage. By contrast, the use of classical tools such as taxonomy, biochemistry and microbial physiology could still be very effective in pre-selecting potential pigment producers. The taxonomical aspect of screening microorganisms is often de-emphasized in patents describing the structure elucidation of bioactive secondary metabolites used for drug leads and as pigments. For example, the producer of the red pigment Arpink Red™ (Table 2) is claimed to be produced by *Penicillium oxalicum* var. armeniaca, a *nomen nudum* (the variety was never described), but from the description in the patent [26] we are of the opinion that the fungus is most likely misidentified as belonging to the genus *Penicillium*. The isolate (CCM 8242, unavailable for the scientific community) is described as having yellow-gold coloured conidia with a diameter of 15–20 μm and 'short' mycelium of a light green colour. None of these characteristics has ever been seen in a *Penicillium* species [12], suggesting that both genus and species identification is probably incorrect. This problem of misidentification is a vital issue for several reasons. For example, if the pigment producer is to be used directly in foods, as in case of some *Monascus* strains for red rice production, mycotoxin production can be a serious problem. In the case of *P. oxalicum*, this species produces a yellow toxic pigment, secalonic acid D [29], and thus cannot be used directly in foods. Moreover, the species grows well at 37 °C [12] and is likely to be a human pathogen, posing a severe threat to the safety of workers in the factory producing the pigment. Along similar lines, we have recently found that *Penicillium marneffei* produces large amounts of extracellular red pigments and one of the pigments was identified as monascorubramine, the red *Monascus* pigment (Figure 1d) (JC Frisvad *et al.*, unpublished). Even though this species produces no known mycotoxins, it is one of the most dangerous human pathogens known [30]. By contrast, on screening the remaining species in the *Penicillium* subgenus *Biverticillium*, which includes *P. marneffei*, we found several other

**(Figure 1 Legend)** The chemical structures of existing naturally derived colorants and potential non-carotenoid fungal pigments. **(a)** Typical naturally derived pigments: chlorophyll a, anthrocyanin, β-carotene, astaxanthin and betanin. **(b)** Carminic acid, the anthraquinone of cochineal. **(c)** Fungal anthraquinones. **(d)** Monascus pigments. **(e)** Potential non-carotenoid fungal pigments.

**Table 2**

**Some promising non-carotenoid fungal pigments as potential food colorants.**

| Fungal source | Pigment | Colour | Comments | Ref |
|---|---|---|---|---|
| **Ascomycetes** | | | | |
| *Monascus* spp. | Monascorubrin<br>Rubropunctatin<br>Monascin<br>Ankaflavin<br>Monascusones | Orange<br>Orange<br>Yellow<br>Yellow<br>Yellow | Well-known pigments of the Orient, authorized in Japan, heat- and pH-stable, give rise to water-soluble red pigments on reacting with amino acids in the media | [21–23] |
| **Anamorphic Ascomycetes** | | | | |
| *Epicoccum nigrum* | Flavipin<br>Orevactaene<br>Unknown | Yellow<br>Orange<br>Yellow | Water-soluble, antioxidant, high colouring power, reported to stimulate astaxanthin production in yeast | [38–40] |
| *Paecilomyces sinclairii*[a] | Unknown | Red at pH 3–4, violet at pH 5–9 and pink at pH 10–12 | Light-stable, high production by submerged cultivation, chemical characterization is needed | [41,42] |
| *Penicillium herquei*, *Roesleria hypogea* and *Penicillium atrovenetum* | Atrovenetin<br>Herqueinone<br>Norherqueinone<br>Several others | Yellow<br>Red<br>Red<br>Bluish green | Atrovenetin is an antioxidant and might exert a dual functionality as a functional food additive, because it potentiates the antioxidant activity of tocopherol (likely mechanism: regeneration of tocopherol by hydrogen donation) | [43–45] |
| *Penicillium oxalicum* var. *armeniaca* | Arpink Red™ | Dark red | Commercially produced, pH- and heat-stable, patented in more than 120 countries | [26–28] |
| *Penicillium purpurogenum*[b] | Purpurogenone<br>Mitorubrin<br>Mitorubrinol | Orange-yellow<br>Yellow<br>Orange to red | Characteristic extracellular red-pink pigment depending on media | [46,47] |
| *Penicillium persicinum* | Unknown | Reddish pink | High amount of exogenous pigment, not yet characterised | [48] |
| *Penicillium fagi* | Unknown | Greenish blue | Mostly trapped in mycelium, uncharacterised | [49] |

[a]Insect pathogen, correct name *Isaria sinclairii.* [b]According to the present classification.

producers of these red pigments. These species neither produced mycotoxins nor were likely to be pathogenic, as they could not grow at 37 °C. Thus, not only the excreted colour but the taxonomic knowledge of fungi would add considerably to the success of the screening process.

Our opinion is that screening for fungal pigments should be based on intelligent screening concepts [31••] where *a priori* knowledge on updated fungal taxonomy and species-specific metabolite profiles can be used to reduce the chances of including pathogenic strains and/or toxin producers from a diverse set of pigment-producing fungi. It would also ensure the correct identification of fungi at the species level to prevent possible mishaps.

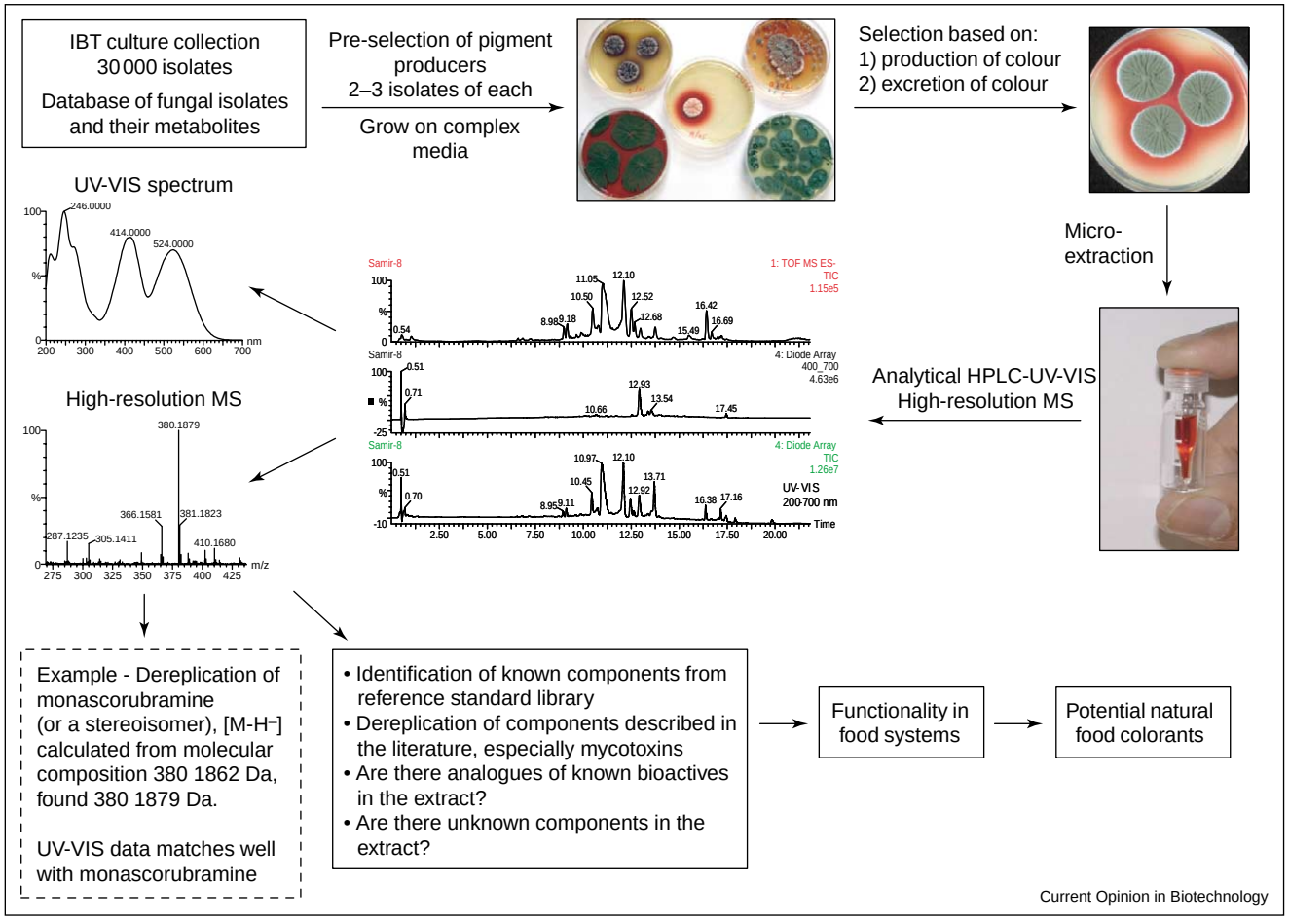
## Intelligent screening: a more rational approach

Prior knowledge of the production of any toxic metabolite by the industrial producer cannot be overlooked. For example, the mycelial food product Quorn™ is produced by *Fusarium venenatum* [32], which also produces a potent cytotoxic metabolite called 4,15-diacetoxyscirpenol [33]. Such knowledge helps in the hazard analysis and safe design of the production process. In the case of pigments, such knowledge could be used to engineer improved biosynthetic pathways by selectively controlling the production of pigments and uncoupling their formation from the synthesis of toxins, as illustrated in the case of *Monascus ruber* [34].

To screen a large collection of potential pigment-producing fungal strains (Figure 2), the initial step should be to use one of the various metabolomic tools to cluster strains based on, for instance, the production of the same group of metabolites [31••]. One of the fastest and most effective profiling methods employs mass spectrometry — fungal extracts are injected directly into a mass spectrometer applying the electrospray ionization technique [35]. Using this method it was demonstrated that strains representing the entire subgenus *Penicillium* in the genus *Penicillium* could be grouped in very good accordance with existing taxonomic knowledge [36•].

With more than 15 000 fungal metabolites already known, it is an immense task to determine whether or not a detected fungal metabolite has already been described;

**Figure 2**




Schematic presentation of the intelligent screening and identification of fungal pigments as potential natural food colorants. Pigment producers are initially preselected from a culture collection such as the IBT fungal culture collection at the Center for Microbial Biotechnology, Lyngby Denmark. Selection is made on the basis of colour production or excretion, and microextraction is used to prepare a sample for analysis. Analytical HPLC analysis is employed to identify coloured compounds. The central panel depicts three HPLC chromatograms (from top): total ion chromatogram (m/z 100–900 nm) from negative ion electrospray, UV-VIS chromatogram of 400–700 nm showing coloured compounds, and UV-VIS chromatogram from 200–700 nm also showing non-coloured compounds. Identification of a pigment (e.g. monascorubramine) is shown on the left-hand side, where the mass of the deprotonated molecular ion $[M-H]^-$, is used to calculate the molecular composition of $C_{23}H_{27}NO_4$ and combined with the entire UV-VIS spectrum. Comparison with the UV-VIS spectral data from the original description of the compound can be used for dereplication and so on.

this task is referred to as dereplication. The use of UV-visible minimum and maximum absorption data and mass spectra is one of the easiest ways to identify coloured compounds such as pigments and can be useful in dereplication. With recent advances in data handling methods and chemoinformatics analyses, such as expert systems for the structure elucidation of natural products [37], we believe that it should be possible to perform a more systematic and automated computer-assisted search of full UV spectra. This would not only lead to the identification of already known compounds in large numbers of chromatographic data files, but in combination with mass spectrometry could reveal new pigments with similar chromophores to those already known (Figure 1a).

## Conclusions

The current trend in society for 'natural' ingredients has stimulated interest in exploring novel means and sources for the biotechnological production of food colorants. In this regard, exploring fungal chemical diversity is a worthwhile route for the identification of novel pigments. An intelligent screening approach for water-soluble pigments that is partly based on chemotaxonomy will provide a platform for the future construction of cell factories for the production of natural food colorants.

Nevertheless, fungal pigments are considered to be alien to food, particularly in societies where their use is not

traditional. Moreover, investments required to carry out the necessary toxicology testing for the safety of such food additives is a major impediment. On the other hand, the unreliable supply of agricultural sources of colour tips the balance in favour of such readily available natural sources. If imperative toxicological testing is carried out, fungal pigments could be accepted by the current consumer. This is supported by the fact that fungal food colorants are now commercially available. Further research using new technologies suggests a promising future for this field of study.

## Acknowledgements

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# *Chapter 3*

*Screening Strategies and Tools towards Identification of Potentially Safe Promising Polyketide Pigment Producing Fungal Cell Factories*

# Chapter 3: Screening Strategies and Tools towards Identification of Potentially safe Promising Polyketide Pigment Producing Fungal Cell Factories

This chapter is a major part of the manuscript "**Identification of potentially safe promising fungal cell factories for the production of polyketide natural food colorants using chemotaxonomic rationale**".

Sameer AS Mapari, Anne S. Meyer, Ulf Thrane, and Jens C. Frisvad.

*Microbial Cell Factories* 2009, **8**:24

The published form of the full manuscript can be found in Appendix A. For a good flow of the thesis, full manuscript is not presented here.

## 3.1 Background

### 3.1.1. Intelligent screening approach

Intelligent screening concept makes use of *a priori* knowledge on updated fungal taxonomy and species specific metabolite profiles (chemotaxonomic perspective) in order to reduce the chances of including pathogenic strains and/or toxin producers from a diverse set of industrially useful metabolite producing fungi. The taxonomic knowledge also provides information about the ecology and thus, this gives a clue to look for a right species in a right habitat. This could be very useful, for instance to look for non-toxigenic strains as soil isolate of *Aspergillus flavus* is toxigenic but plant isolates are not. The knowledge about the species also gives a way to maximize the biodiversity by studying a few isolates from a number of species rather than studying a mammoth number of unidentified isolates. The chemotaxonomic approach also allows finding alternative producers of a specific metabolite as well as organisms producing related compounds, *e.g.* looking at species from similar habitats or the same species series.

Intelligent screening involves such effective tools as metabolomic tools *eg*. mass spectrometry, to cluster stains based on, for instance, the production of the same group of metabolites. The use of UV-vis spectra and mass spectra ensures one of the easiest ways to identify compounds. This is very useful for dereplication; which is to find out whether or not a detected fungal metabolite has already been described.

The other important tool that forms an integrated part of intelligent screening is the use of algorithms as quick and effective data handling tools in order to perform a more systematic and automated computer-assisted search of full UV-vis spectra.

## 3.2. Introduction

With an appropriately selected fungus the fungal natural colorants; unlike flowering plants, plant cell or algal sources of colorants, could be produced in high yields by using the available cultivation technology without potential genetic manipulation as tougher legislation and skeptical attitude of consumers make it almost impossible for the genetically modified food. The controlled cultivation of pigment producing fungi in bioreactors has the potential to compete with any other means of production and can potentially provide unlimited quantities of colourings provided that imperative toxicological studies are carried out. However, a first requirement is that the potential fungus producing the pigment(s) is non-toxigenic under the given broader range of production conditions and is non-pathogenic to humans. The ability of filamentous fungi to co-produce mycotoxins along with industrially useful extrolites, e.g. as in case of citrinin produced by some of the pigment and statins producing *Monascus* species, is a major bottleneck in their approval by the legislative authorities. Some of the pigment producers, for instance, *Penicillium marneffei*, could even be human pathogens (1). Different cultivation media have been shown to induce production of different pigments (2); a systematic evaluation of the effect of different media components on pigment production – and/or a better understanding of the factors inducing pigment production in fungi – is currently highly warranted in order to optimize the pigment production.

Thus, it is of utmost importance to address the question of how to rationally select a promising fungal pigment producer considering the enormous chemical and biodiversity of fungi. Moreover, the molecular and genetic basis for the polyketide pigment biosynthesis in fungi have not yet been examined in detail leaving genomic approaches for screening unfeasible at this point of time.

In the light of this, a comprehensive list, based on chemotaxonomy, of a majority of the polyketide pigment producing ascomycetous fungi, their pigment composition, and the toxigenic potential with a list of known coloured as well as uncoloured toxic metabolites, is provided. Based on taxonomic knowledge, focus was given on pigment producing ascomycetous fungi belonging to *Penicillium* subgenus *Biverticillium* that produced yellow-orange-red pigments while human pathogenic and mycotoxigenic strains belonging to the same group were deselected. Chemotaxonomic selection and/or de-selection approach was exemplified in two Penicillia *viz. Penicillium aculeatum* and

*Penicillium crateriforme* grown on 5 different complex solid media to identify potential pigment producer that produced known pigments with or without mycotoxin. The aim of this study was to prove the pertinent use of chemotaxonomic trait of metabolite profiling by powerful tools as HPLC-DAD-MS to come up with promising polyketide pigment producing cell factories that are neither known to be human pathogens nor to produce any known mycotoxins. The ultimate goal is to establish such potentially safe fungal cell factories for the production of polyketide natural colorants.

## 3.3 Materials and methods

**Pre-selection of fungi, media, and cultivation conditions**

All fungal isolates used in this study were procured from the IBT Culture Collection at Center for Microbial Biotechnology, Technical University of Denmark, Kgs. Lyngby, Denmark. The fungal isolates were listed by the IBT numbers. *Penicillium aculeatum* IBT 14259 and *P. crateriforme* IBT 5015 was cultivated on five different solid media *viz*; Yeast extract sucrose (YES) agar; Malt extract agar (MEA), Oatmeal (OAT) agar, Potato dextrose (PD) agar and Czapek-Dox yeast autolysate (CYA) agar (3). The cultures were incubated in the dark at 25 $^{o}$C for 7 days.

**Extraction of fungal pigments**

Extraction was carried out by a modified version of the micro-scale extraction method (4), where 6 mm plugs were extracted ultrasonically with solvent containing ethyl acetate, dichloromethane, and methanol in a ratio of 3:2:1 (v/v) with 1% formic acid. The extract was evaporated to dryness in a rotational vacuum concentrator (RVC; Christ Martin, Osterode, Germany). Residue was redissolved in 400 µl methanol, in an ultrasonic bath (Branson 2510, Kell-Strom, Wethersfield, USA) for 10 minutes, and filtered through a 0.45 µl PTFE syringe filter (SRI, Eatontown, NJ, USA). This extract was used for chromatographic analysis.

**Chromatographic analysis**

High-resolution LC-DAD-MS was performed on an Agilent HP 1100 liquid chromatograph (LC) system with a photodiode array detector (DAD) and a 50 × 2 mm i.d., 3 µm, Luna C 18 II column (Phenomenex, Torrance, CA). The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, U.K.) with a Z-spray electrospray ionization (ESI) source, and a LockSpray

probe and controlled by the MassLynx 4.0 software. MS system was operated in the positive ESI mode using a water-acetonitrile gradient system starting from 15% acetonitrile, which was increased linearly to 100% in 20 minutes with a holding time of 5 minutes. The water was buffered with 10 mM ammonium formate and 20 mM formic acid and the acetonitrile with 20 mM formic acid. The instrument was tuned to a resolution > 7000 (at half peak height). The method is well established at our research center for the metabolite profiling of filamentous fungi grown on solid media. It is described by Nielsen *et al*. (5).

## 3.4 Results

### Chemotaxonomic selection /de-selection for potential polyketide pigment producing ascomycetous fungi

**Table 3.1** and **3.2** provide a comprehensive list of ascomycetous fungi that produce pigments in visible amounts and a majority of these fungal species (belonging to the genera *Penicillium, Fusarium, Alternaria, Aspergillus* and partially *Epicoccum*) have been metabolically profiled for mycotoxin production at our research center. This *a priori* knowledge on potential mycotoxin production and the evaluation of pathogenic potential on the basis of BioSafety Level (BSL) classification (6) formed the basis of our pre-selection/deselection of pigment producers. Strains of the species belonging to *Penicillium* subgenus *Biverticillium* (**Table 3.1**) were known to produce copious amounts of yellow, orange and red pigments on solid media; one of the representative red pigment producers is shown in **Figure 3.1.** These strains of the species were chosen to be studied. *A priori* deselection of strains of the 4 species *viz. P. islandicum, P. marneiffei, P. variabile,* and *P. rugulosum* including the teleomorph *Talaromyces macrosporus* was carried out; the strains being either pathogenic and/or mycotoxigenic. Chemotaxonomic selection/deselection approach was exemplified in strains of *P. crateriforme,* and *P. aculeatum* where by *P. crateriforme* served as a positive control in which the presence of mycotoxin was known *a priori* but the pigment was still uncharacterized. The chromatographic analyses of two representative pigment extracts that illustrate our selection/de-selection approach are presented in **Figures 3.2 - 3.4. Figure 3.2 (A- F)** depicts the extracted ion chromatogram (m/z 269.12) obtained by positive ESI chromatography using authentic standard of rugulovasine A and B. The pigment extracts of *Penicillium crateriforme* IBT 5015 grown on CYA (**Figure 3.2B**), MEA (**Figure**

**3.2C**), PD (**Figure 3.2D**), OAT (**Figure 3.2E**), and YES (**Figure 3.2F**) media were found to be positive for the presence of rugulovasine A and B. **Figure 3.3 (A-F)** depicts the absence of rugulovasine A and B in the pigment extracts of *Penicillium aculeatum* IBT 14259 grown on YES (**Figure 3.3B**), PD (**Figure 3.3C**), OAT (**Figure 3.3D**), MEA (**Figure 3.3E**), and CYA (**Figure 3.3F**). Both of these Penicillia produced a well known orange *Monascus* pigment, monascorubrin, in CYA as shown in **Figure 3.3, B and C** by means of the extracted ion chromatogram (m/z 383.19) and mass spectrum. Since *Penicillium crateriforme* IBT 5015 produced rugulovasine A and B, in addition to rubratoxin, and spiculisporic acid (7), it was deselected, while *Penicillium aculeatum* IBT 14259 was selected.

**Figure 3.1 *Penicillium purpurogenum* IBT 11180 on YES agar after 7 days of incubation showing extracellular pigment production.**

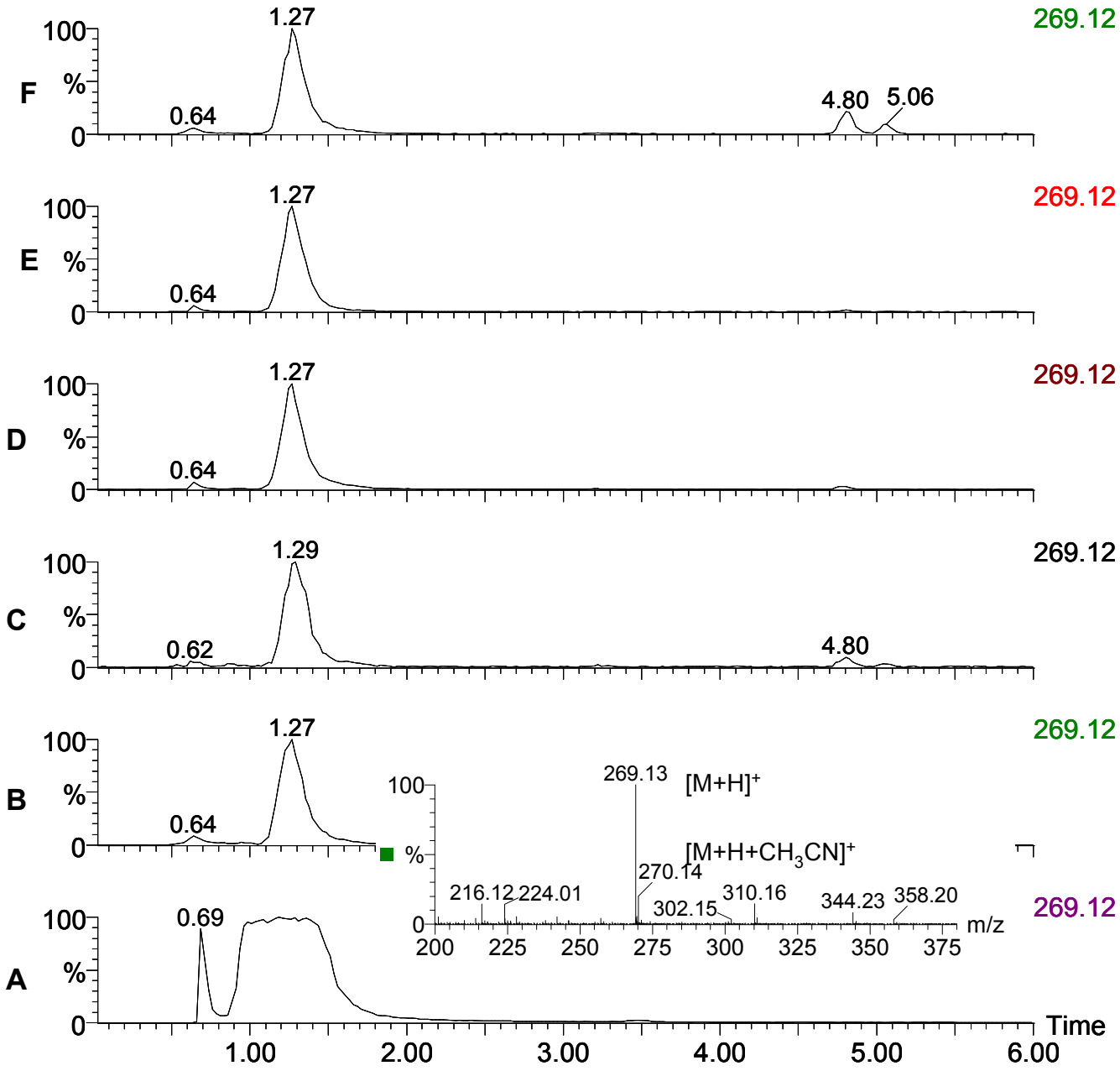


**Figure 3.2** Extracted ion chromatogram (m/z 269.12) of standard rugulovasine A and B and pigment extracts of *Penicillium crateriforme* IBT 5015 grown on 5 different solid media depicting the presence of rugulovasine A and B with its mass spectrum.

**3.2A**. Standard rugulovasine A and B; **3.2B**. Pigment extract from CYA; **3.2C**. Pigment extract from MEA; **3.2D**. Pigment extract from PD; **3.2E**. Pigment extract from OAT; **3.2F.** Pigment extract from YES

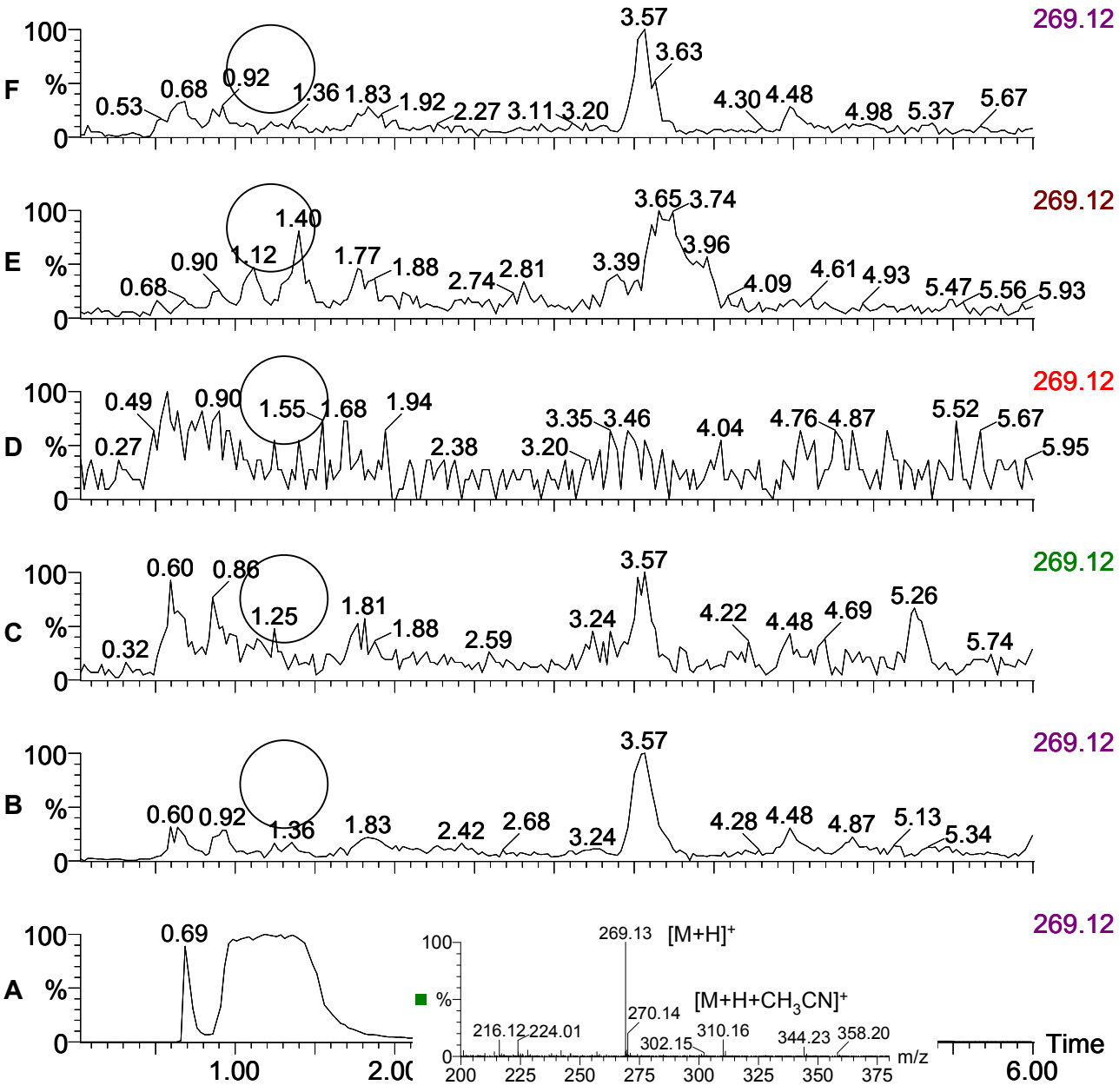


**Figure 3.3** - Extracted ion chromatogram (m/z 269.12) of standard rugulovasine A and B and pigment extracts of *Penicillium aculeatum* IBT 14259 grown on 5 different solid agar media depicting the absence of rugulovasine A and B with its mass spectrum.

**3.3A**. Standard rugulovasine A and B; **3.3B**. Pigment extract from YES; **3.3C**. Pigment extract from PD; **3.3D**. Pigment extract from OAT; **3.3E**. Pigment extract from MEA; **3.3F.** Pigment extract from CYA.

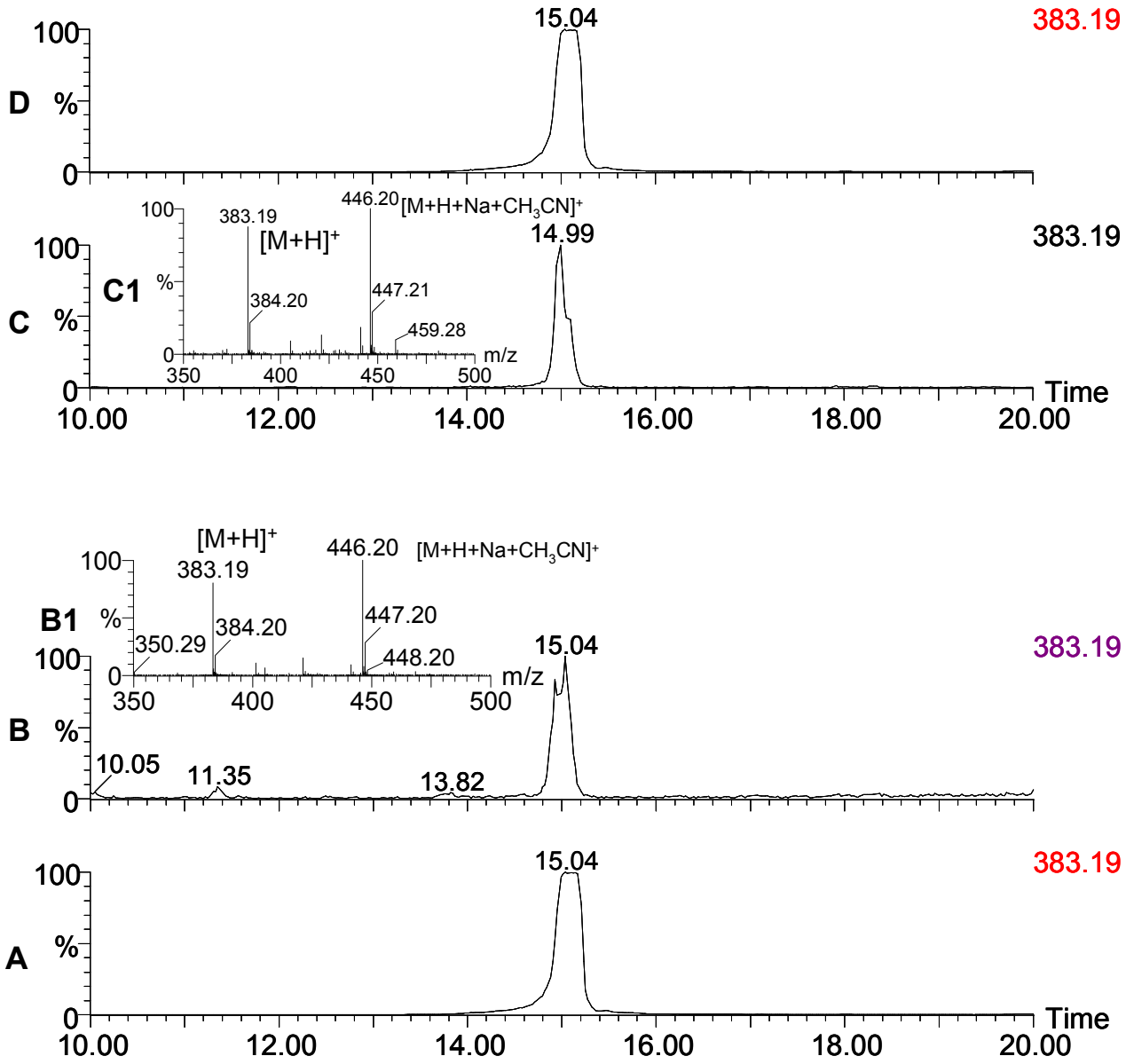


**Figure 3.4** - Extracted ion chromatogram (m/z 383.19) of standard monascorubrin and pigment extracts of *Penicillium aculeatum* IBT 14259 and *Penicillium crateriforme* IBT 5015 on CYA depicting the presence of monascorubrin with its mass spectrum.

Bottom panel **A**. standard monascorubrin; **B**. pigment extract of *Penicillium crateriforme* IBT 5015 on CYA; **B1**. mass spectrum of monascorubrin; Top panel **C**. pigment extract of *Penicillium aculeatum* IBT 14259 on CYA ; **C1**. mass spectrum of monascorubrin **D**. standard monascorubrin.

## 3.5 Discussion

**Chemotaxonomic selection /de-selection for potential polyketide pigment producing ascomycetous fungi**

The strains of the species (underlined in **Table 3.1** and **3.2**) are yet to be investigated as potential production strains on different solid and in liquid media. Some of them may be promising as potentially safe cell factories for pigments with an array of different colors indicating that a lot more fungal biodiversity is yet to be explored for the discovery of novel sources of natural colorants. *Epicoccum nigrum* (**Table 3.2**) has been evaluated for pigment production (8), and *Penicillium herquei* (**Table 3.1)** has been partially evaluated (data unpublished). They could be potential cell factories for natural green-yellow to yellow colorants. Biosynthetically, a majority of pigments produced by filamentous fungi are polyketide-based (some may involve polyketide-amino acid mixed biosynthesis) and involve complex pathways catalysed by iterative type I polyketide synthases as exemplified in case of pigments produced by *Monascus ruber* (9). A complete knowledge about the biosynthetic pathway of polyketide pigments including the extensively studied *Monascus* pigments is not yet available. Also, genomic approaches of selection of potential polyketide pigment producers may be not as useful as chemotaxonomic approaches at this point of time when none of the fungal polyketide pigment producers are fully genome sequenced yet. Moreover, the problem of annotating correct gene sequences is not to be overlooked especially due to the variation in the domain of polyketide synthases; two or more polyketide synthases are involved in the biosynthesis of such complex secondary metabolites as pigments. Chemotaxonomy uses secondary metabolite profiles of filamentous fungi as the secondary metabolites have a differentiation capability at a genus and species level [10]. This has been used successfully and resulted in a lot of *a priori* knowledge about filamentous fungi – their bio- and chemical diversity, ecological niche, the species-specific metabolite profiles, and optimal media and growth conditions for secondary metabolites (including pigments) production [11]. Phylogeny as a taxonomic tool, whereby the partial sequences of the household genes such as ß-tubulin are used, on the other hand, cannot predict the functional trait of the organisms as described by Samson *et al.* [12]. Therefore, chemotaxonomic selection would form an essential element of high throughput screening programmes as the use of *a priori* knowledge of species-specific metabolite/pigment and/or mycotoxin profiles ensures a quick and efficient way of selecting potentially safe pigment producers from a vast bio- and chemodiversity of filamentous fungi.

**Table 3.1 Comprehensive list of polyketide pigment producing *Penicillium* and *Aspergillus* species and their known teleomorphs.** The *a priori* species-specific major pigment and/or toxic metabolite profiles and the BioSafety Level (BSL) classification of the fungal species are highlighted and form the basis for selection/de-selection of the species as a potential source of pigment production.

| Fungal species[1] | Pigment composition (colour). Toxic colored compounds in bold | Major known mycotoxic uncolored metabolites | BioSafety Level (BSL) classification[2] [6] | Comment[3] | Ref. |
|---|---|---|---|---|---|
| ***Penicillium* subgenus *Penicillium*** | | | | | |
| *P. atramentosum* | Uncharacterized dark brown | Roquefortine C<br>Rugulovasine A & B | unknown | No | [13] |
| *P. atrosanguineum* | Phoenicin (red)<br>Uncharacterized yellow and red | unknown | unknown | TBI | [14] |
| *P. atrovenetum* | Atrovenetin (yellow)<br>Norherqueinone (red) | beta-Nitropropionic acid | unknown | No | [15] |
| *P. aurantiogriseum* | Uncharacterized | Nephrotoxic glycopeptides<br>Penicillic acid<br>Verrucosidin | 1 | No | [13] |
| *P. brevicompactum* | Xanthoepocin (yellow) | Botryodiploidin<br>Mycophenolic acid | 1 | No | [13] |
| *P. chrysogenum* | Sorbicillins (yellow)<br>Xanthocillins (yellow) | Roquefortine C | 1 | No | [13] |
| *P. citrinum* | Anthraquinones (yellow)<br>**Citrinin** (yellow) | | 1 | No | [16] |
| *P. cyclopium* | **Viomellein** (reddish-brown)<br>**Xanthomegnin** (orange) | Penicillic acid | unknown | No | [13] |
| *P. discolor* | Uncharacterized | Chaetoglobosin A, B & C | unknown | No | [13] |
| *P. echinulatum* | Uncharacterized (yellow) | Territrems | unknown | No | [13] |
| *P. flavigenum* | Xanthocillins | unknown | unknown | TBI | [13] |
| *P. freii* | **Viomellein** (reddish-brown)<br>**Vioxanthin**<br>**Xanthomegnin** (orange) | | unknown | No | [13] |
| *P. herquei* | Atrovenetin (yellow)<br>Herqueinones (red and yellow) | unknown | unknown | Yes | [17] |

| | | | | | |
|---|---|---|---|---|---|
| *P.oxalicum* | Arpink red™- anthraquinone derivative (red)<br>**Secalonic acid D** (yellow) | | unknown | No | [18, 19] |
| *P. paneum* | Uncharacterized (red) | Botryodiploidin<br>Patulin<br>Roquefortine C | unknown | No | [13] |
| *P. persicinum* | Uncharacterized (Cherry red) | Roquefortine C | unknown | No | [13] |
| *P. viridicatum* | **Viomellein** (reddish-brown)<br>**Vioxanthin**<br>**Xanthomegnin** (orange) | Penicillic acid<br>Viridic acid | unknown | No | [13] |
| ***Talaromyces*** **(anamorph *Penicillium* subgenus *Biverticillium*)** | | | | | |
| *T. macrosporus* | Mitorubrin (yellow) | Duclauxin<br>Islanditoxin | unknown | No | [20] |
| *P. aculeatum* | Uncharacterized | | unknown | Yes | [un-pub-lished] |
| *P. crateriforme* | Uncharacterized | Rubratoxin<br>Rugulovasine A &B<br>Spiculisporic acid | unknown | No | [21] |
| *P. funiculosum* | Uncharacterized | | unknown | Yes | [22] |
| *P. islandicum* | **Emodin** (yellow)<br>**Erythroskyrin** (orange-red)<br>**Luteoskyrin** (yellow)<br>**Skyrin** (orange) | Cyclochlorotine<br>Islanditoxin<br>Rugulosine<br>Rugulovasine A & B | unknown | No | [21] |
| *P. marneffei* | Mitorubrinol<br>Monascorubramine (purple-red)<br>Purpactin<br>Rubropunctatin (orange)<br>**Secalonic acid D** (yellow) | | 3 | No | [un-pub-lished] |
| *P. pinophilum* | Uncharacterized | | unknown | Yes | [22] |
| *P. purpurogenum* | Mitorubrin (yellow)<br>Mitorubrinol (orange-red)<br>PP-R (purple red)<br>Purpurogenone (yellow-orange) | | 1 | Yes | [2, 21, 23] |
| *P. rugulosum* | **Rugulosin** (yellow) | | 1 | No | [7, 21] |
| *P. variabile* | **Rugulosin** (yellow) | | unknown | No | [7, 21] |
| ***Eurotium*** **(anamorph *Aspergillus* subgenus *Aspergillus*)** | | | | | |
| *E. amstelodami* | Auroglaucin (orange)<br>Erythroglaucin (red)<br>Flavoglaucin (yellow)<br>**Physcion** (yellow) | Echinulin | 1 | No | [7] |
| *E. chevalieri* | Auroglaucin<br>Erythroglaucin<br>Flavoglaucin<br>**Physcion** (yellow) | Echinulin | 1 | No | [7] |

| | | | | | |
|---|---|---|---|---|---|
| *E. herbariorum* | Aspergin (yellow)<br>Flavoglaucin (yellow)<br>**Physcion** (yellow) | Echinulin | 1 | No | [7] |
| ***Aspergillus* section *Circumdati*** | | | | | |
| *A. ochraceus* | **Viomellein** (reddish-brown)<br>**Vioxanthin**<br>**Xanthomegnin** (orange) | Ochratoxin A<br>Penicillic acid | 1 | No | [24] |
| *A. melleus* | **Rubrosulphin** (red)<br>**Viomellein** (reddish-brown)<br>**Viopurpurin** (purple)<br>**Xanthomegnin** (orange) | | unknown | No | [24] |
| *A. sulphureus* | **Rubrosulphin** (red)<br>**Viomellein** (reddish-brown)<br>**Viopurpurin** (purple)<br>**Xanthomegnin** (orange) | | unknown | No | [24] |
| *A. westerdijkiae* | **Rubrosulphin** (red)<br>**Viomellein** (reddish-brown)<br>**Viopurpurin** (purple)<br>**Xanthomegnin** (orange) | Ochratoxin A<br>Penicillic acid | unknown | No | [24] |
| ***Aspergillus* section *Nigri*** | | | | | |
| *A. niger* | Flavioline (orange-red),<br>Nnaptho-γ-pyrones (yellow) | Fumonisins<br>Ochratoxin A | 1 | No | [25] |
| *A. sclerotioniger* | Uncharacterized yellow | Ochratoxin A | unknown | No | [25] |
| ***Emericella***<br>**(anamorph *Aspergillus* subgenus *Nidulantes*, section *Nidulantes* and section *Versicolores*)** | | | | | |
| *Em. falconensis* | Falconensins C-H (yellow)<br>Falconensones (Yellow)<br>Zeorin (yellow) | unknown | unknown | TBI | [26] |
| *Em. purpurea* | Epurpurins A-C (yellow) | unknown | unknown | TBI | [27] |
| *A. versicolor* | **Sterigmatocystin** (yellow) | | 1 | No | [28] |

[1] Underlined species are yet to be explored as pigment producers.
[2] BSL-1: saprobes or plant pathogens occupying non-vertebrate ecological niches, or commensals. Infections are coincidental, superficial, and non-invasive or mild. BSL-3: pathogens potentially able to cause severe, deep mycoses in otherwise healthy patients.
[3] Keys to selection; Yes: preselected as a possible source of pigments, No: not selected as a possible source of pigments, TBI: to be investigated as a possible source of pigments.

**Table 3.2 Selected ascomycetous fungi and their species-specific polyketide pigment and/or toxic metabolite profiles.** The *a priori* major metabolite profiles and the BioSafety Level (BSL) classification of the fungal species are highlighted and form the basis for selection/de-selection of the species as a potential source of pigment production.

| Fungal species[1] | Pigment composition (colour). Toxic colored compounds in bold | Major known mycotoxic uncolored metabolites | BioSafety Level (BSL) classification[2] [8] | Comment[3] | Ref. |
|---|---|---|---|---|---|
| *Fusarium acuminatum* | Antibiotic Y (yellow)<br>Aurofusarin (red) | Enniatins<br>Moniliformin | unknown | No | [29] |
| *F. avenaceum* | Antibiotic Y (yellow)<br>Aurofusarin (red) | Enniatins<br>Moniliformin<br>2-amino-14,16-di-methyloctadecan-3-ol | unknown | No | [29] |
| *F. culmorum* | Aurofusarin (red)<br>Fuscofusarin (yellow)<br>Rubrofusarin (red) | Butenolide<br>Fusarin C<br>Trichothecenes<br>Zearalenone | unknown | No | [29] |
| *F. fujikuroi* | Bikaverin (red)<br>Norbikaverin (red)<br>O-demethylanhydrofusarubin (red) | Fumonisins<br>Fusaric acid<br>Gibberellins<br>Moniliformin | 1 | No | [30] |
| *F. graminearum* | Aurofusarin (red)<br>Rubrofusarin (red) | Butenolide<br>Fusarin C<br>Trichothecenes<br>Zearalenone | unknown | No | [29] |
| *F. oxysporum* | 2,7-dimehoxy-6-(acetoxyethyl)juglone (yellow)<br>Bikaverin (red)<br>Bostrycoidin (red)<br>Nectriafurone (yellow)<br>Norjavanicin (red)<br>O-methyl-6-hydroxynorjavanicin (yellow)<br>O-methylanhydrofusarubin (orange-red)<br>O-methylfusarubin (red)<br>O-methyljavanicin | Fumonisins<br>Fusaric acid<br>Moniliformin | 2 | No | [30] |
| *F. poae* | Aurofusarin (red) | Enniatins<br>Fusarin C<br>Trichothecenes | unknown | No | [29] |
| *F. sambucinum* | Aurofusarin (red) | Trichothecenes | unknown | No | [29] |
| *F. solani* | Fusarubin (red)<br>Isomarticins (red)<br>O-ethylfusarubin (red)<br>O-methyldihydrofusarubin | | 2 | No | [30] |

| | | | | | |
|---|---|---|---|---|---|
| | (red) | | | | |
| *F. sporotrichioides* | Aurofusarin (red)<br>Lycopene | Enniatins<br>Trichothecenes | unknown | No | [29] |
| *F. stilboides* | Antibiotic Y (yellow)<br>Aurofusarin (red)<br>Nectriafurone (yellow) | Enniatins | unknown | No | [un-pub-lished] |
| *F. tricinctum* | Antibiotic Y (yellow)<br>Aurofusarin (red) | Fusarin C<br>Moniliformin | unknown | No | [29] |
| *F. venenatum* | Aurofusarin (red)<br>Rubrofusarin (red) | Trichothecenes | unknown | No | [29] |
| *F. verticillioides* | Fusarubin<br>O-demethylfusarubin<br>O-methyljavanicin<br>O-methylsolaniol (orange-red) | Fumonisins<br>Fusaric acid<br>Moniliformin | unknown | No | [30] |
| *Alternaria dauci* | Uncharacterized (red) | unknown | 1 | TBI | [31] |
| *Alt. porri* | Altersolanol A (yellow-orange) | unknown | 1 | TBI | [31] |
| *Alt. solani* | Altersolanol A (yellow-orange) | unknown | 1 | TBI | [31] |
| *Alt. tomatophila* | Altersolanol A (yellow-orange) | unknown | unknown | TBI | [31] |
| *Cladosporium cladosporioides* | Calphostins A, B, C, D, I (red) | unknown | 1 | TBI | [32] |
| *Cordyceps unilateralis* | 3,5 8-TMON* (red)<br>4-O-methyl erythrostominone (red)<br>Deoxyerythrostominol (red)<br>Deoxyerythrostominone (red)<br>Epierythrostominol (red)<br>Erythrostominone (red) | unknown | unknown | TBI | [33] |
| *Curvularia lunata* | Chrysophanol (red)<br>Cynodontin (bronze)<br>Helminthosporin (maroon) | unknown | 1 | TBI | [16] |
| *Drechslera* spp. | Catenarin (red)<br>Cynodontin (bronze)<br>Helminthosporin (maroon)<br>Tritisporin (redish-brown) | unknown | unknown | TBI | [16] |
| *Epicoccum nigrum** | Chromanone (yellow)<br>Epicoccarines A & B<br>Epicocconone (fluorescent yellow)<br>Epipyridone (red)<br>Flavipin (brown)<br>Isobenzofuran derivatives (yellow to brown)<br>Orevactaene (yellow) | unknown | unknown | Yes | [2, 34, 35] |
| *Paecilomyces sinclairii* | Uncharacterized (red) | unknown | unknown | TBI | [36] |

| | | | | | |
|---|---|---|---|---|---|
| **Polyketide pigment producer of Asia** | | | | | |
| *Monascus pilosus* | **Citrinin** (yellow) | | 1 | Banned in the EU & the US | [37] |
| *M. purpureus* | Ankaflavin (yellow)<br>**Citrinin** (yellow)<br>Monascin<br>Monascorubramine<br>Monascorubrin<br>Rubropunctamine (purple-red)<br>Rubropunctatin (orange) | Monascopyridine A & B | 1 | Banned in the EU & the US | [37] |
| *M. ruber* | Ankaflavin (yellow)<br>**Citrinin** (yellow)<br>Monascin<br>Monascorubramine<br>Monascorubrin<br>Rubropunctamine (purple-red)<br>Rubropunctatin (orange) | | 1 | Banned in the EU & the US | [37] |

[1] Underlined species are yet to be explored as pigment producers, * also known to produce carotenoids.
[2] BSL-1: saprobes or plant pathogens occupying non-vertebrate ecological niches, or commensals. Infections are coincidental, superficial, and non-invasive or mild. BSL-2: Species principally occupying non-vertebrate ecological niches, but with a relatively pronounced ability to survive in vertebrate tissue. In severely immuno-compromised patients they may cause deep opportunistic mycoses. Also pathogens causing superficial infections are in this category.
[3] Keys to selection; Yes: preselected as a possible source of pigments, No: not selected as a possible source of pigments, TBI: to be investigated as a possible source of pigments.

The purpose of the present work was to demonstrate the chemotaxonomic selection and de-selection approach in the pigment producing fungi whereby specific mycotoxin production was shown in the producer or the non-producer in addition to the *a priori* knowledge of their mycotoxin and pigment profile. Thus, the starting point of our selection approach was the use of chemotaxonomic knowledge about the polyketide pigment producing fungi (**Tables 3.1** and **3.2**). We used this as a key and came up with a handful of pigment producers that are worth exploring as potentially safe fungal cell factories for polyketide pigment production.

## 3.6 Conclusions

In conclusion, it was shown that the use of chemotaxonomic tools and *a priori* knowledge of fungal extrolites is a rational approach towards selection of potentially safe polyketide natural colorant producing fungal cell factories considering the enormous chemical diversity and biodiversity of ascomycetous fungi. The chemotaxonomic rationale could also be very handy for the selection of potentially safe fungal cell factories

not only for polyketide pigments but also for the other industrially important polyketides, the molecular and genetic basis for the biosynthesis of which has not yet been examined in detail.

## 3.7 References


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# *Chapter 4*

## *Colorimetric Characterization for Comparative Analysis of Fungal Pigments and Natural Food Colorants*

# Chapter 4: Colorimetric Characterization for Comparative Analysis of Fungal Pigments and Natural Food Colorants

This chapter is based on the published manuscript "Colorimetric Characterization for Comparative Analysis of Fungal Pigments and Natural Food Colorants"

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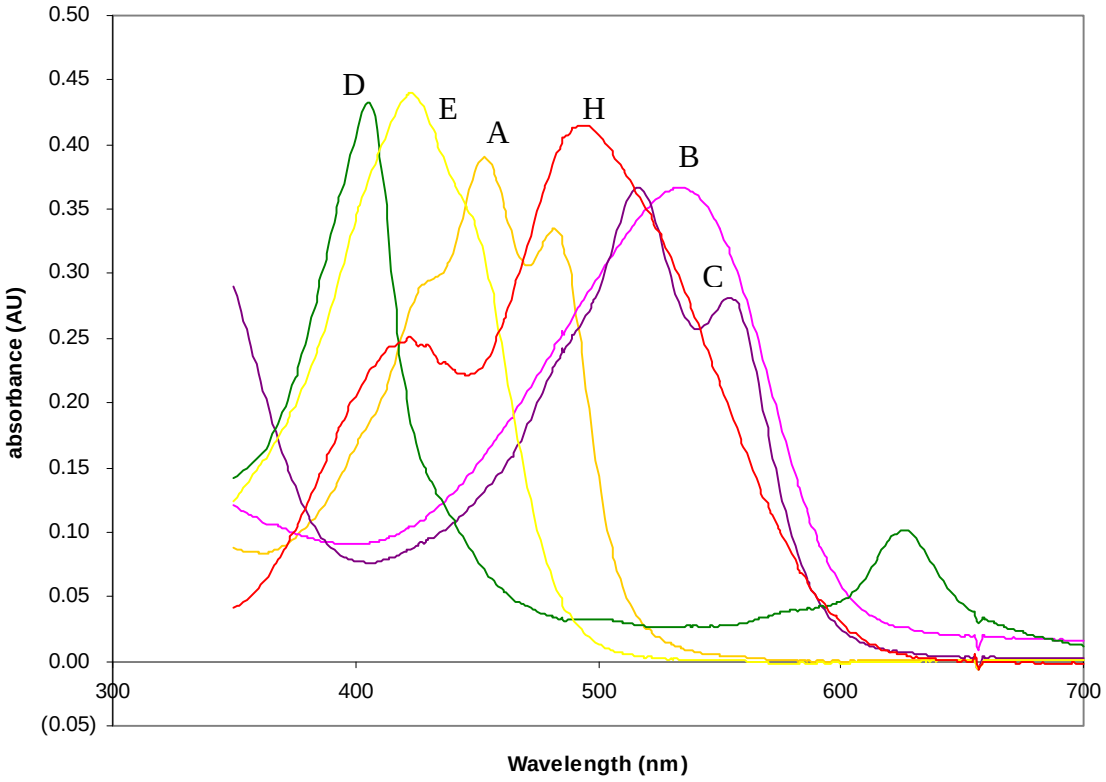
## 4.1 Background

### 4.1.1 Qualitative investigations of physical spectra and the absorption maxima do not always provide the correct color: An illustration

**Figure 4.1** Absorption spectral bands of some commercially available reference food colorants; A, Annatto; B, Beet root; C, Acid-stable carmine; D, Chlorophyllin; E, Turmeric; H, *Monascus* Red 3




The role of shape of a given absorption band in determining the visually observed color can be explained clearly by looking at the spectrum of beet root [B] (**Figure 4.1**). Beet

root, also a red colorant, has an absorption maximum at 536 nm. From the absorption maximum alone, one would expect its visual color to be crimson red. However, the CIELAB color space which takes into account the wavelength-dependent sensitivity of the human eye, places it in the blue-violet-pink region as indicated by its hue angle value of 352 (**Table 1 and Figure 1 in the following manuscript**). It has been reported that the sensitivity of the human eye varies over the entire visible region and finds its maximum at 555 nm (**Reference 21 in the following manuscript**). In this case, the eye overrates the light contribution arising from the extended slope around 572 nm. Absorption at this specific wavelength alone would have produced a brilliant bluish red color, while the absorption at 536 nm produces red color. As a result, the beet root color appears violet-red. Therefore, the shape of a given absorption band, in addition to the absorption maxima, is a key factor in determining the visually observed color. Also, the observed pink-red color of acid-stable carmine [C] (**Table 1 and Figure 1 in the following manuscript**) can be explained not only by its additional local maximum in the yellow-green region (560 nm) but also by its characteristic absorbance pattern over the entire visible spectrum as shown in (**Figure 4.1**). Along similar lines, the difference in the yellowness of annatto [A] and turmeric [E] (**Figure 4.1**) can be explained. The absorption spectra of annatto [A] showed two local absorption maxima, one being the absolute maximum at 458 nm and the other at 484 nm respectively besides a shoulder at 436 nm, while turmeric [E] had an absolute maximum at 424 nm (**Figure 4.1**). Therefore, annatto is deeper yellow in color than turmeric The green color of chlorophyllin [D] was characterized by two local absorption maxima approximately at 400 and 625 nm, in the violet and the orange region respectively (**Figure 4.1)**. With respect to colorants, a qualitative inspection, thus, does not allow one to decide whether the color originates from a single green colorant or from a mixture of a blue and a yellow one.

In the light of the above-mentioned illustration on the shortcoming of a qualitative approach, colorimetric characterization was carried out using a quantitative approach based on CIELAB color space which takes into account the sensitivity of the human eye towards lights of different wavelengths and intensities.

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# Colorimetric Characterization for Comparative Analysis of Fungal Pigments and Natural Food Colorants

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Exogenous pigments produced by ascomycetous filamentous fungi belonging to the genera *Penicillium*, *Epicoccum*, and *Monascus*, preselected based on chemotaxonomic knowledge, have been extracted and characterized by quantitative colorimetry. The color characteristics of the fungal extracts were compared to water soluble natural colorants derived from sources currently in use. The tested fungal extracts also included some commercially available *Monascus* colorants. The *a** values for the fungal extracts were found to be both positive and negative, the *b** values were found to be positive, while the hue angles of the fungal color extracts ranged from 40 to 110 indicating the color distribution of fungal extracts over the red−orange−yellow region of the CIELAB color space. The fungal extracts exhibited additional color hues in the red spectrum and similar hues in the yellow spectrum as compared to the reference natural colorants. They were also found to be similar or brighter in terms of chroma to some of the reference natural colorants. Principal component analysis was performed to group and distinguish different colors based on the *a** and *b** values. The fungal color extracts could be grouped in accordance with the similarity or difference in the color to those of the existing natural colorants. The diversity of colors was not only found among different fungal genera and/or species but also within the same species on changing the media. There was a marked change in the color composition of the extracts resulting in relatively different hues. Our results, thus, indicate that there exists pigment-producing genera of ascomycetous fungi other than *Monascus* that produce color shades in the red and the yellow spectra in addition or similar to reference colorants. These color shades could add to the color palette of the natural colorants currently in use. In addition, the multivariate approach in distinguishing and classifying the colorants was shown to be a very useful tool in colorimetric comparison of colorants.

**KEYWORDS: Fungal pigments; colorimetry; natural colorants; *Monascus* pigments; multivariate analysis**

## INTRODUCTION

The utilization of natural pigments in foodstuff has been increasing in recent years due to the marketing advantages of employing natural ingredients and due to consumer concerns about eventual harmful effects of synthetic pigments; Fast Green dye, widely used as a food colorant, has been shown to be an immunotoxic agent (*1*). Currently, natural pigments are derived from sources like plants (*2*−*4*), insects (*5*), and microorganisms (*6*−*10*). The latter is gaining much attention outside of the food industry in Europe and the United States because of the stability of the pigments produced (*11*), the possibility for in-house production, and the availability of cultivation technology that can be optimized for higher yields (*12*, *13*). The ultimate aim is the production of pigments by microbial biotechnology that would be independent of the external supply of agricultural raw materials and climatic conditions. The use of filamentous fungi, such as *Monascus*, as sources of food colorants has a long-term history in the Orient but is still forbidden in the West except for the successful production of $\beta$-carotene from the fungus *Blakeslea* (*14*). One unsuccessful attempt of a food colorant from fungi in the West was of the anthraquinone-based red fungal pigment Arpink Red (*14*, *15*), which received a temporary 2 year approval for distribution in the Czech Republic from 2004 until May 5, 2006. We believe that exploring fungal chemical diversity by a chemotaxonomic approach, whereby the preselection of a nontoxigenic and a nonpathogenic strain is ensured, is a worthwhile route for the identification of potential pigment-producing fungi as sources of color leads for the food industry (*15*). Most of the available literature about pigment-producing fungi for food use points toward *Monascus*, which produces pigments that are good food colorants because they are stable in the pH range of 2−10, heat stable to autoclaving, and exhibit different colors of yellow to red (*16*, *17*). There is a report (*18*) on characteristics and stability of pigments produced by *Monascus anka* where it was found that

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these pigments were stable under UV and fluorescent light but were very sensitive to sunlight. The same study also brought out that tartaric and citric acids were detrimental to the *Monascus* pigments and that the copper ions showed the most deleterious effect on color change of these pigments. Besides *Monascus* pigments, several characteristic noncarotenoid pigments are produced by filamentous fungi that exhibit a unique structural and chemical diversity with an extraordinary ranges of colors (*19*). Noncarotenoid pigments of filamentous fungi are secondary metabolites, many of which are structurally diverse polyketides. These polyketide pigments are often complex compounds that are at least partially synthesized by multifunctional enzymes called polyketide synthases (*20*). The structures of polyketides are known to have loose $\pi$-electrons as they often contain polyunsaturated functionality, i.e., ring systems, one or more carbonyl groups, carboxylic acid, and ester or amide functional groups exhibiting characteristic UV−vis spectra. Therefore, we assume that these polyketides may serve a potential source of new chromophores (color leads) that can be promising for food use.

Even though color is a perceptual phenomenon, a quantitative description of color is a key factor for both scientific investigations and applications of colorants in food products. Qualitative investigations of physical spectra and the absorption maxima do not always provide the correct color (*21*). Therefore, a more quantitative approach is needed to describe a color. One of the ways color can be described quantitatively is the use of extinctions of colored solutions at their respective absorption maxima according to the Lambert−Beer law. Another widely adopted way of describing a color is based on stimuli generated in the human eye by visible light of various wavelength and intensities. The so-called CIE (Commission International de l'Eclairage) system is based on the fact that light reflected from any colored surface can be visually matched by an additive mixture of the three primary colors: red, green, and blue (*21*).

Recently, there have been a couple of reports on describing the color characteristics of *Monascus* pigment derivatives (*22*, *23*) and their enhancement of photostability to improve their scope for industrial application. Moreover, the successful production of $\beta$-carotene from the fungus *Blakeslea* over a decade and the recent temporary authorization of a fungal food colorant in the Czech Republic (*14*, *15*) have put a large incentive to characterize the color of fungal pigments more than *Monascus* pigments for future food use. However, the lack of coproduction of volatile compounds in significant amounts that may alter the organoleptic characteristics of the food and toxicity are some of the key factors that need to be tested for the food application of fungal pigments.

In light of this, we used chemotaxonomic tools and a priori knowledge of fungal metabolites to preselect some potential pigment-producing, nonpathogenic, and nontoxigenic ascomycetous fungi. The present paper describes the color characteristics of fungal pigment extracts from such fungi using quantitative colorimetry in order to test our hypothesis that the hues and chroma of fungal pigments in the red and the yellow spectra are comparable to the existing natural food colorants; hence, fungi may serve as a new source of natural colorants for food use. We focus on the color of fungal pigments to emphasize their similarity or differences to the existing commercially available counterparts used as references in our study by multivariate data analysis based on their CIELAB color characteristics. The pigment compositions of some of the representative crude color extracts were also analyzed chromatographically to understand the difference in their color hues, and some of the components were identified by high-resolution liquid chromatography−diode array detection−mass spectrometry (LC-DAD-MS).

## MATERIALS AND METHODS

**Standard Colorants.** Eight colorants, viz. annatto, beet root, carthamus, lutein, natural carotene, turmeric, carminic acid, and acid-stable carmine (7-$\beta$-D-glucopyranosyl-9,10-dihydro-5-amino-3,6,8-trihydroxy-1-methyl-9,10-dioxo-2-anthracenecarboxylic acid) (*5*), were kindly delivered by Chr. Hansen A/S (Hørsholm, Denmark). Four *Monascus* colorants were obtained from different suppliers from China and Japan as follows: *Monascus* Red 1 was provided by Feng sheng (China), *Monascus* Red 2 was provided by Yaegaki (Japan), *Monascus* Red 3 was provided by Riken Vitamin Co. Ltd. (Japan), and *Monascus* Yellow was provided by Kelongbio (China). The colorants were dissolved directly in distilled water (pH 6.8−7.0), except in the case of natural carotene where drops of acetone were added to completely dissolve the colorant, and were subsequently used for colorimetric characterization.

**Fungi, Media, Cultivation Conditions, and Name Codes.** All fungal isolates used in this study were procured from the IBT Fungal Culture Collection at BioCentrum-DTU, Technical University of Denmark (Kgs. Lyngby, Denmark). The fungal isolates were listed by the IBT numbers. All fungi were cultivated on three different solid media, namely, yeast extract sucrose (YES) agar, potato dextrose (PD) agar, and Czapek−Dox yeast autolysate (CYA) agar (*24*). Three of the pigment-producing fungi that exogenously produced intense coloration on solid media were also grown in liquid medium, Czapek−Dox (CZ) broth (*24*), with an initial pH adjusted to 6.5. The cultures were incubated in the dark at 25 °C for 7 days. Liquid cultivations were performed in 500 mL of baffled Erlenmeyer flasks containing 100 mL of the medium (CZ) at 150 rpm on a rotary shaker at 25 °C for 7 days. **Table 1** represents the identity of reference colorants and the source of fungal extracts viz. the name of fungi, the IBT number, and the media used for their incubation. Specific name codes have been assigned to each of the color extracts, and hence forth, they have been referred to by these codes in all of the figures and tables.

**Extraction of Fungal Pigments.** Extraction was carried out by a modified version of the microextraction method (*25*), where 6 mm plugs were extracted in two steps in a 2 mL vial for 30 min, first using 1 mL of ethyl acetate with 0.5% formic acid to break open the cell wall and extract relatively apolar metabolites. The extract so obtained was then transferred to a new 2 mL vial and evaporated in vacuo. The second extraction was performed using 1 mL of methanol or isopropanol based on our preliminary results indicating maximum pigment extraction from the specific pigment-producing fungus (data unpublished). Because the exact chemical nature of pigments varied from fungus to fungus, it was necessary to use the appropriate solvent for the specific strain. By doing so, we could extract the maximum color. However, the same solvent system was used to extract for the same strains cultured in different media. The second extract was then added to the vial with the residue from the previous extraction. It was then evaporated in a rotational vacuum concentrator (RVC; Christ Martin, Osterode, Germany). The residue was redissolved in 400 $\mu$L of methanol, in an ultrasonic bath (Branson 2510, Kell-Strom, Wethersfield, CT) for 10 min and filtered through a 0.45 $\mu$L PTFE syringe filter (SRI, Eatontown, NJ). A part of this extract was used for chromatographic analysis, and the rest was used for quantitative colorimetry. In case of the liquid medium CZ, where the pigment was mostly diffused in the media, the color extract was obtained as per the method used by Jung et al. (*22*).

**Colorimetry.** The absorbance values of reference colorants as well as the fungal color extracts were adjusted to 0.40 ± 0.04 at their respective absorption maxima with purified water, obtained from a Milli-Q system (Millipore, Bedford, MA), as a diluent. The absorption maxima were determined by scanning the extracts for their absorption spectra over the range of 350−700 nm, using a spectrophotometer (Agilent HP 8453, Agilent Technologies, Palo Alto, CA). The same extracts were then used for determining CIELAB color coordinates using Chromameter (Minolta CT 310, Konica Minolta, Mahwah, NJ).

**Table 1.** CIELAB Color Coordinates of Reference Water-Soluble Natural Colorants and Fungal Color Extracts

| sample code | sample/fungal name | IBT no. | media | *L* | *a** | *b** | hue angle[a] | chroma[b] |
|---|---|---|---|---|---|---|---|---|
| A | acid-stable carmine | | | 85.62 | 28.24 | –0.78 | 358.42 | 28.25 |
| B | carminic acid | | | 93.94 | 9.22 | 29.18 | 72.5 | 30.60 |
| C | beet root | | | 83.36 | 30.30 | –4.13 | 352.24 | 30.58 |
| D | annatto | | | 98.16 | –9.98 | 39.76 | 104.9 | 40.99 |
| E | turmeric | | | 99.68 | –10.57 | 27.79 | 110.82 | 29.73 |
| F | carthamus | | | 100.00 | –5.98 | 15.49 | 111 | 16.60 |
| G | lutein | | | 93.92 | –2.88 | 30.48 | 95.3 | 30.61 |
| H | natural carotene | | | 98.01 | –6.62 | 40.10 | 99.3 | 40.64 |
| I | *Monascus* red 1 | | | 87.27 | 16.34 | 24.73 | 56.6 | 29.64 |
| J | *Monascus* red 2 | | | 86.92 | 18.14 | 20.35 | 48.3 | 27.26 |
| K | *Monascus* red 3 | | | 85.62 | 21.89 | 18.20 | 39.7 | 28.46 |
| L | *Monascus* yellow | | | 94.97 | –3.99 | 37.87 | 95.9 | 38.07 |
| M | *P. purpurogenum* chemotype II | 11180 | CYA | 87.85 | 10.69 | 22.64 | 64.8 | 25.03 |
| M1 | *P. purpurogenum* chemotype II | 11180 | YES | 85.81 | 15.90 | 16.54 | 46.2 | 22.94 |
| M2 | *P. purpurogenum* chemotype II | 11180 | CZ | 85.01 | 19.72 | 22.38 | 46.62 | 29.83 |
| N | *Penicillium aculeatum* | 14263 | CYA | 87.83 | 11.95 | 14.48 | 50.5 | 18.77 |
| N1 | *P. aculeatum* | 14263 | YES | 88.44 | 10.51 | 21.76 | 64.3 | 24.16 |
| O | *P. purpurogenum* | 3951 | YES | 83.80 | 12.78 | 17.36 | 53.64 | 21.56 |
| O1 | *P. purpurogenum* | 3951 | CZ | 84.9 | 16.21 | 22.06 | 53.8 | 27.37 |
| P | *Monascus ruber* | 7904 | MEA | 91.07 | 10.76 | 11.80 | 47.7 | 15.96 |
| Q | *Penicillium atrovenetum* | 5990 | YES | 97.50 | –3.65 | 14.03 | 104.5 | 14.49 |
| Q1 | *P. atrovenetum* | 5990 | CZ | 98.55 | –3.08 | 9.35 | 108.23 | 9.84 |
| R | *Penicillium herquei* | 21731 | YES | 97.47 | –3.99 | 15.61 | 104.3 | 16.11 |
| R1 | *P. herquei* | 21731 | CZ | 98.17 | –4.16 | 11.71 | 109.5 | 12.42 |
| S | *E. nigrum* | 7571 | YES | 97.12 | –6.11 | 24.04 | 104.2 | 24.8 |
| T | *E. nigrum* | 7232 | YES | 94.87 | –6.59 | 32.29 | 101.5 | 32.95 |
| U | *E. nigrum* | 7802 | YES | 96.69 | –7.83 | 32.16 | 103.6 | 33.09 |
| V | *E. nigrum* | 7901 | YES | 96.48 | –7.94 | 29.89 | 104.8 | 30.92 |
| W | *E. nigrum* | 41028 | CYA | 97.85 | –7.71 | 28.81 | 104.9 | 29.82 |
| W1 | *E. nigrum* | 41028 | PD | 97.68 | –8.98 | 31.67 | 105.8 | 32.91 |

[a] Hue angle ($h_{ab}$) = $\tan^{-1}$ ($b^*/a^*$). [b] Chroma ($C$) = $[(a^*)^2 + (b^*)^2]^{1/2}$.

The CIELAB colorimetric system was interpreted as follows: $L^*$ indicates lightness read from 0 (black) to 100 (white). The positive $a^*$ value indicates the red color while the negative $a^*$ value represents the green color. Similarly, positive and negative $b^*$ values indicate the yellow and the blue colors, respectively. Chroma values denote the saturation or purity of color. In a color wheel, values close to the center, at the same $L^*$ value, indicate dull or gray colors, whereas values near the circumference represent vivid or bright colors. Hue angle values represent the degree of redness, yellowness, greenness, and blueness; the maximum is at 0, 90, 180, and 270, respectively.

**Chromatographic Analysis.** High-resolution LC-DAD-MS was performed on an Agilent HP 1100 LC system with a DAD and a 50 mm × 2 mm i.d., 3 $\mu$m, Luna C 18 II column (Phenomenex, Torrance, CA). The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, United Kingdom) with a *Z*-spray electrospray ionization (ESI) source and a LockSpray probe and controlled by the MassLynx 4.0 software. The MS system was operated in the positive ESI mode using a water−acetonitrile gradient system starting from 15% acetonitrile, which was increased linearly to 100% in 20 min with a holding time of 5 min or starting from 5% acetonitrile for 2 min and increasing to 100% in 18 min and keeping it for 5 min. The water was buffered with 10 mM ammonium formate and 20 mM formic acid, and the acetonitrile was buffered with 20 mM formic acid. The instrument was tuned to a resolution >7000 (at half peak height). The method is well-established at our Center as described by Nielsen et al. (*26*).

**Analysis of LC-DAD-MS Data.** The presence of PP-R was detected in $ESI^+$ from the first scan function of the reconstructed ion chromatograms of *m*/*z* 426.4288 $[M + H]^+$ and confirmed by the fragments *m*/*z* 448.3817 $[M + H + Na]^+$ and 489.3471 $[M + H + Na + CH_3CN]^+$, and the presence of orevactaene was detected in $ESI^+$ from the same scan function as *m*/*z* 613.2708 $[M + H]^+$ and confirmed by the fragments *m*/*z* 595.3037 $[M + H - H_2O]^+$ and 635.2318 $[M + H + Na]^+$.

**Principal Component Analysis (PCA) Analysis.** The colorimetric data (comprised of $a^*$ and $b^*$ color coordinates) were analyzed by PCA using The Unscrambler 7.6 on Windows NT, 2000 and XP. Prior to statistical analyses, all variables were standardized to mean zero and variance one.

## RESULTS AND DISCUSSION

**Comparative Analyses of Colorimetric Values of Fungal Color Extracts and Water-Soluble Natural Colorants.** The red and the yellow commercially available water- and oil-soluble natural colorants were used as references for comparing the red and the yellow fungal crude extracts. The absorbance for all colorants in the present study was adjusted to a value of 0.40 ± 0.04; as a result, the lightness values for all colorants were similar and varied in the range from 83 to 100 (**Table 1**). The $a^*$ values for the fungal extracts were found to be both positive, ranging from 11 to 20, and negative, ranging from −3 to −9, while the $b^*$ values were only positive with values ranging from 9 to 32 (**Table 1)**. The hue angles of all of the fungal extracts ranged from 46 to 110. This indicated the color of fungal extracts to be red to orange and yellow to light green-yellow on the basis of the CIELAB color system as explained in the Materials and Methods. To compare the hue angles and chroma, i.e., the purity or saturation of a color, of the red and the yellow colorants, two-dimensional polar scatter plots were made at the same lightness level of 87 ± 3 and 97 ± 2 for the two groups of colorants, respectively.

*Hues.* The hue angle values for the fungal extracts [M], [M1], [M2], [N], [N1], [O], [O1], and [P] ranged from 46 to 65 (**Table 1)**, signifying orange-red color (**Figure 1**), while the hue angle values of fungal extracts [Q], [Q1], [R1], [S], [T], [U], [V], [W], and [W1] ranged from 101 to 110 (**Table 1)**, signifying yellow to green yellow in color (**Figure 2**). Among the red reference colorants, the hue angle of acid-stable carmine [A] was 358.4, which is close to the hue angle value of beet root

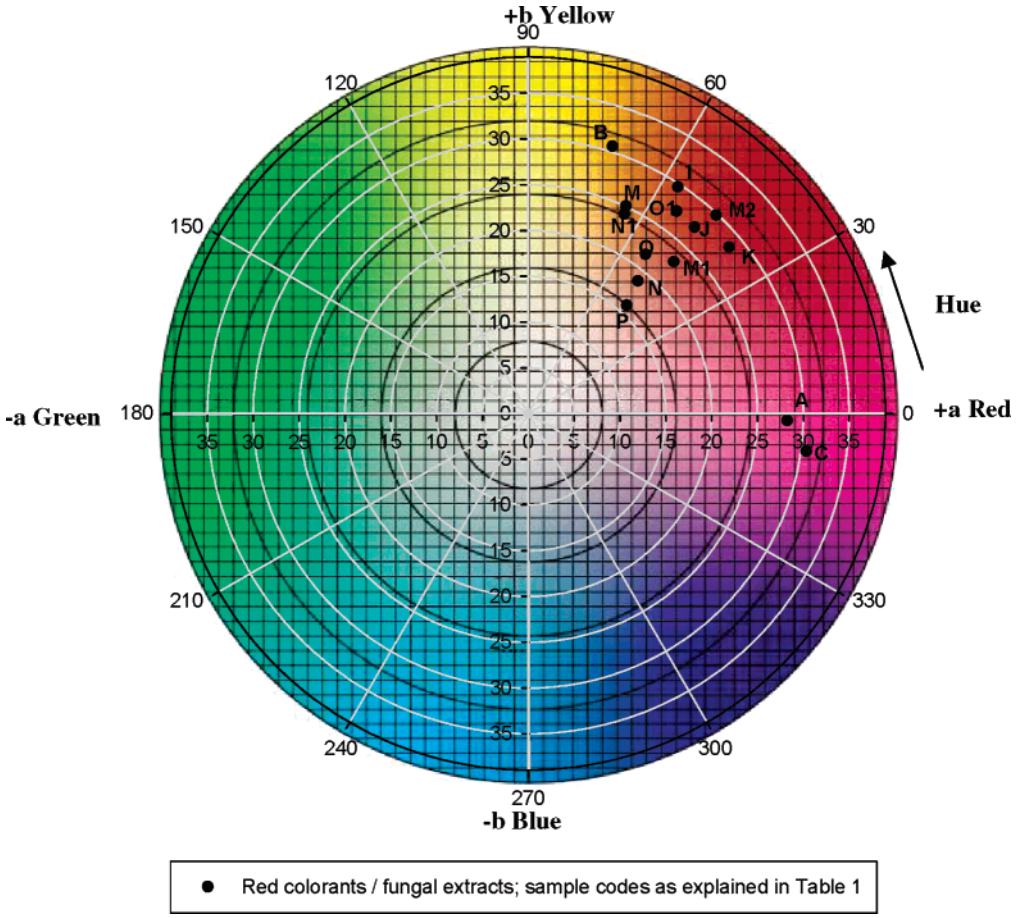


**Figure 1.** Polar scatter plot showing hue angles and chroma of standard red colorants and red fungal extracts at *L* values of 87.4 ± 3.3.

[C], which was 352.2, indicating light blue-red color; and the hue angle of carminic acid [B] was 72.5 indicating light orange color (**Table 1** and **Figure 1**). The commercially available red *Monascus* colorants [I, J, and K] had hue angles in the range of 40−57 (**Table 1**). These three *Monascus* colorants thus represented orange-red hues. Among the yellow reference colorants, the hue angle value of lutein [G] was 95.3 indicating the highest degree of yellowness followed by natural carotene [H] with a hue angle value of 99.3. The other yellow reference colorants viz. annatto [D], turmeric [E], and carthamus [F] had hue angles ranging from 105 to 111, indicating green-yellow color (**Table 1** and **Figure 2**). The red fungal extracts including commercially available *Monascus* colorants showed scarlet red or middle red as compared to the red reference colorants as their hue angles were in the range of 30−65. The color of the red fungal extracts was similar to color hues of the control red and orange *Monascus* pigments reported by Jung et al. (*22*). Thus, it can be inferred that there are several species of Penicillia that produce pigments of similar color hues like that of *Monascus*, and these could be interesting to explore further. Chemodiversity was exhibited among different species and/or chemotypes in terms of color as seen from the different hue angles of extracts [M], [N], and extracts [M1], [N1], and [O], respectively, representing various hues of orange-red color (**Table 1** and **Figure 1**). The diversity of colors was also seen among the same strains but grown on different media as exemplified by the red extracts [M], [M1], [N], and [N1] (**Table 1** and **Figure 1**). Among the yellow fungal extracts, the hue angle of the fungal extract [W] was 104.9, the same as for the reference colorant annatto [D]. Fungal extracts [Q], [S], [U], [V], and [W1] had relatively similar hue angles as compared to annatto [D] (**Table 1** and **Figure 2**). Thus, the yellowness of most of the yellow fungal extracts was comparable to the yellowness of annatto [D] with respect to the hue angle. The fungal extract [R1] was similar to the reference colorant turmeric [E] in terms of hue angle indicating a similar yellowness. The yellowness of the fungal extract [T] was comparable to the yellowness of natural carotene [H] as indicated by their similar hue angles (**Table 1** and **Figure 2**). There are not any previous data available on the CIELAB color characteristics of yellow fungal pigments for comparison, especially in the context of food use. This signifies the contribution of the present findings in this area and also shows that the yellow hues of fungal pigments may also prove to be worth considering for food use.

*Chroma.* Carminic acid [B] and beet root [C] had the highest chroma values of 31 (**Table 1**) representing the brightest color hues closely followed by *Penicillium purpurogenum* chemotype II IBT 11180 on CZ medium [M2], *Monascus* Red 1 [I] with chroma values of 30, and *Monascus* Red 3 [K] with a chroma value of 29 (**Table 1** and **Figure 1**). The fungal extracts [M], [O1], and *Monascus* Red 2 [J] were similar in brightness as acid-stable carmine [A] (**Figure 1**) as their chroma values were found to be in the range of 25−28 (**Table 1**). Fungal extracts [P] and [N] were found to be relatively dull or gray as compared to both reference colorants and other red fungal extracts as their chroma values were in between 15 and 20 (**Figure 1**). Among the yellow colorants, annatto [D] and natural carotene [H] had the highest chroma values of 41 representing the brightest color hues followed by *Monascus* Yellow [L] with a chroma value of 38 (**Table 1** and **Figure 2**). Fungal extracts *Epicoccum nigrum* IBT 7232 on YES [T], *E. nigrum* IBT 7802 on YES [U], *E. nigrum* IBT 7901 on YES [V], *E. nigrum* IBT 41028

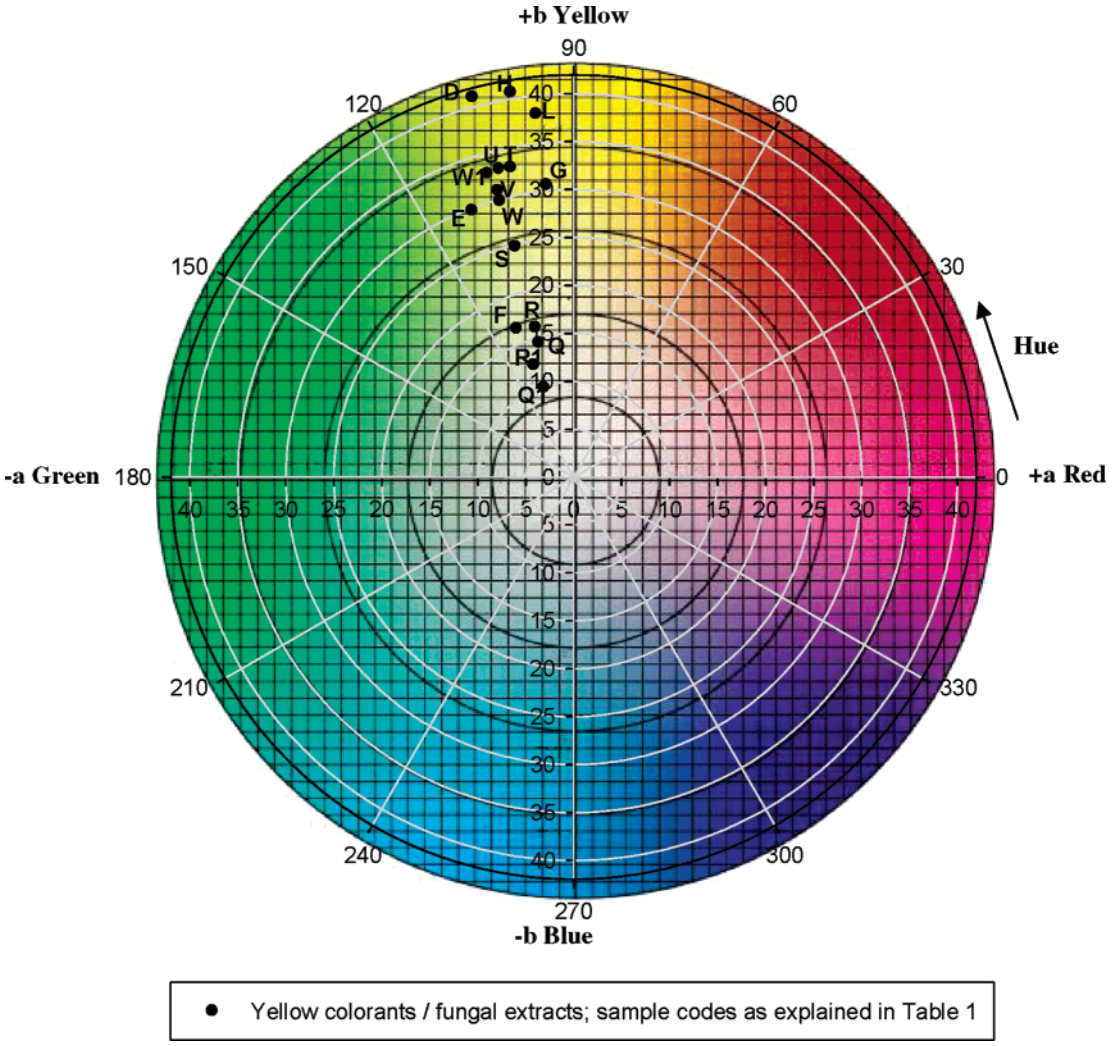


**Figure 2.** Polar scatter plot showing hue angles and chroma of standard yellow colorants and yellow fungal extracts at *L* values of 97.4 ± 1.8.

on CYA [W], and *E. nigrum* IBT 41028 on PD [W1] had chroma values in the range of 30−33 similar to the chroma values of the reference colorants turmeric [E] and lutein [G] and hence the brightness (**Table 1** and **Figure 2**). The chroma value of the yellow fungal extract [R] was comparable to the brightness of the reference colorant carthamus [F], while the fungal extracts [Q], [Q1], and [R1] were relatively dull and gray as compared to the other yellow fungal extracts and the yellow reference colorants as indicated by their lower chroma values ranging from 10 to 15 (**Table 1** and **Figure 2**).

Thus, the colors of fungal extracts in the present study including commercially available *Monascus* colorants provide additional hues in the red spectrum and similar hues in the yellow spectrum as compared to the reference colorants derived from contemporary sources. In terms of chroma, no significant difference was observed between fungal extracts and reference colorants. In some cases, the fungal extracts were found to be similar or brighter than some of the reference colorants, while as compared to some other reference colorants, they were found to be gray or dull.

**Multivariate Analysis.** In order to test the use and validation of a multivariate approach to group and distinguish different colors, PCA analysis was performed using *a** and *b** color coordinates. The red and the yellow fungal extracts formed quite distinct clusters based on *a** and *b** values while the red colorants beet root [C] and acid-stable carmine [A] were seen far to the right in the plot (**Figure 3**). Hence, PCA analysis was clearly able to discriminate these colors from the other red colorants. As discussed earlier, the color hue of beet root and carmine lied in the blue-violet-pink region of the CIELAB color space (**Figure 1**); therefore, it was located far away from the rest of the red colorants. The red fungal extracts clustered around the red *Monascus* colorants, signifying a similar color of the fungal extracts to the commercially available *Monascus* red colorants. The color palette of the fungal extracts including *Monascus* colorants was clearly shown to be in between the color range of the tested red natural colorants [A] and/or [C] and [B] in the red region of the CIELAB color space. In the yellow group of colorants, left-hand side region of the plot, the distinction between the colorants was more visible as the points were well-spread within the group in the same pattern as in the CIELAB color space (**Figures 1** and **2**). The PCA score plot thus exhibited an enlarged view of the points located in the CIELAB color space discriminating the red and the yellow colorants more explicitly by spreading the points in the space across the two components. Clearly, the PCA score plot based on *a** and *b** values (**Figure 3**) discriminates the color characteristics in agreement to that of the CIELAB color space (**Figures 1** and **2**) thus validating the grouping of colorants by the use of multivariate approach.

**HPLC-DAD-MS Analysis of Crude Fungal Color Extracts.** After colorimetric characterization, some representative red and yellow crude fungal extracts were analyzed by a LC system with a DAD coupled to a MS to determine the pigment

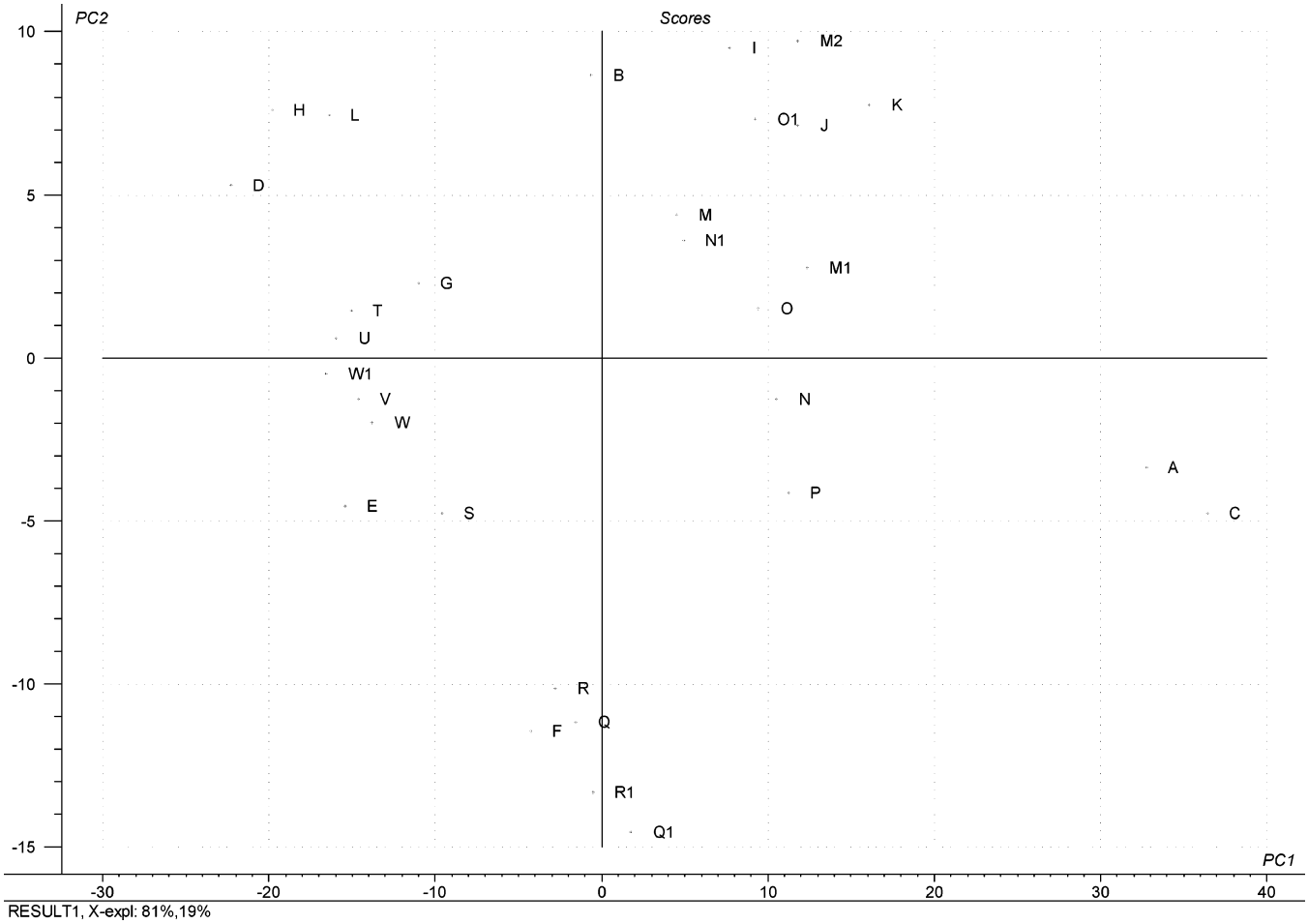


**Figure 3.** PCA score scatter plot showing the classification of colorants based on *a** and *b** values where sample codes are explained in **Table 1**.

composition and identify the color components of the extract. The retention times of only the major colored components detected between 400 and 700 nm contributing ≥10% of peak area (normalized as per the highest peak) were measured. Some of these major color components were identified based on their UV−vis and mass spectra (**Figure 4**). **Figure 5** depicts the structures of the characteristic pigments detected and identified.

*Red Extracts.* The red extracts [M] and [O] showed the presence of a single major component whereas extracts [M1] and [N1] had two components each, and the extract [N] had four different components (**Table 2**). This indicated that the color could result from either a single major color component or a mixture of colored components. In this case, it resulted from a mixture of components, and each of these color components contributed to the final color based on their typical absorption spectra exhibiting the difference in the color hues (M1 and N, N1 in **Figure 1**) in the red spectrum. If the color was due to a single major component, a slight change in the pattern of the absorption spectra including the area under the peak could result in the variation of color hues (M and O in **Figure 1**). However, it must be noted that the variation in the color hue due to the minor colored components in the crude extract was neglected as they were found to be insignificantly present in most of the colored extracts studied. The retention time of major peaks and their absorption spectra were found to be unique (**Table 2**) for each of the red extracts indicating their characteristic color composition. Both of these attributes of the color composition explained the chemodiversity in terms of color among different genera, species, and/or chemotypes also among the same strain grown on different media. The feature that different color hues results on different media is very significant considering the potential biotechnological production of such fungal food colorants. In this case, the cause of this effect might be due to a change in the color composition resulting from a derivative of a parent compound differing only in a slight change in the chromophore and having the same basic polyketide backbone; the results put a large incentive into investigating the metabolic pathways of pigments production in fungi, as such an understanding may lead to possibilities for directing the pathways into desired pigment production and hence a desired hue. One of the major components of the red extract M1 obtained from *Penicillium purpurogenum* chemotype II on YES media was identified to be PP-R, 7-(2-hydroxyethyl)monascorubramine (top panel of **Figure 4**), a structural homologue of the *Monascus* pigments, ankaflavin and monascorubrin. There have been reports where PP-V, a homologue of the red *Monascus* pigment, monascorubramine, and also PP-R were found to be produced by an unidentified *Penicillium* sp. (*27*−*30*). Also, the absorption spectra in the visible region of the major components of red fungal extracts (M, M1, N, N1, and O) from Penicillia (**Table 2**) showed a general pattern comprised of two absorption maxima at 412−430 and 512−528 nm except for the peak 2 in the extract N1 (**Table 2**), which had a single absorption maximum at 462 nm. These values are in agreement with the findings of Jung et al. (*22*), who reported the absorption maximum wavelengths of 422 and 508 for the red *Monascus* pigments monascorubramine and rubropunctamine and of 417−427 and 498−525 nm for their derivatives. Similar absorption spectral patterns with either a single absorption maximum value near 470 nm or two absorption maxima near 410 and 500 nm

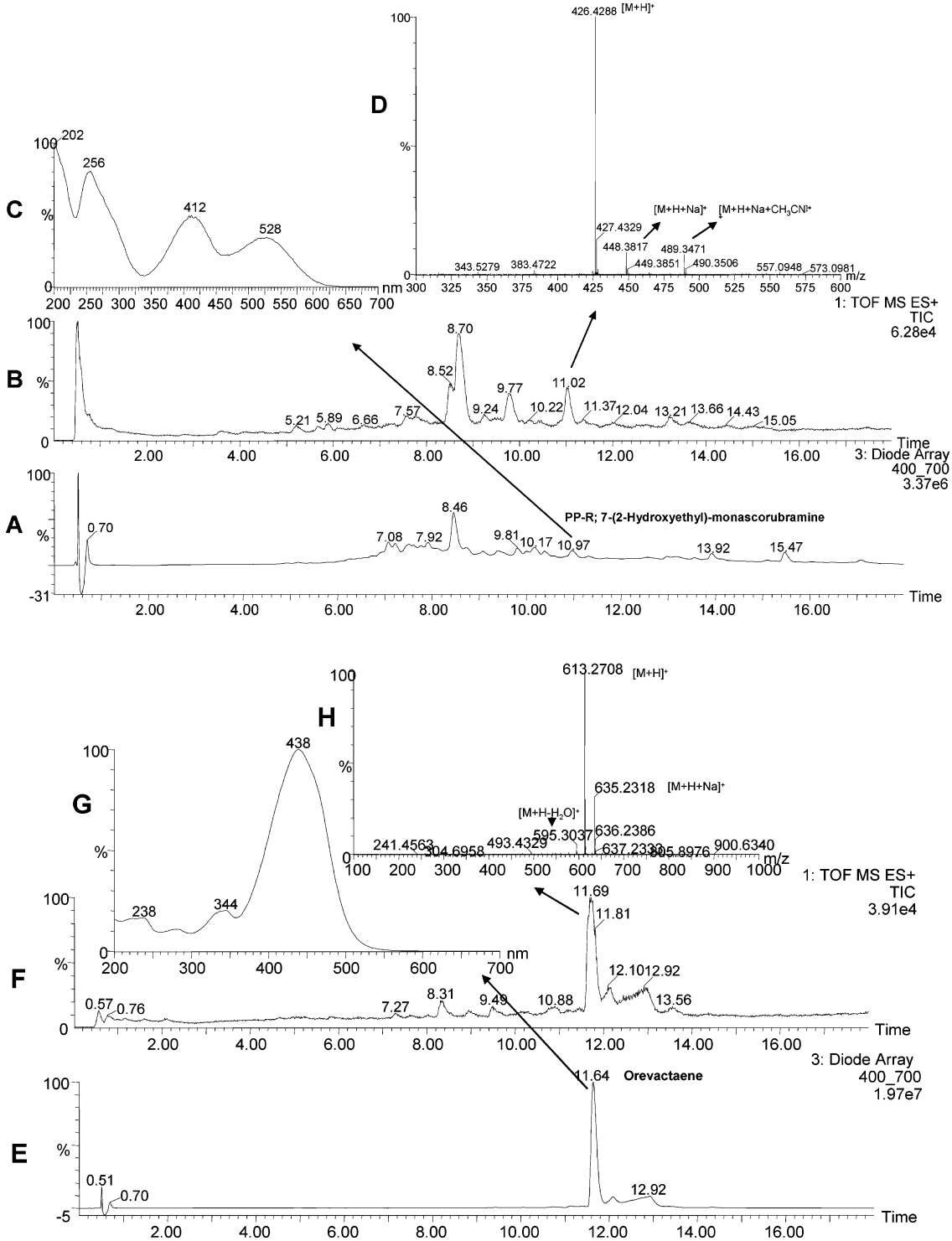


**Figure 4.** LC-UV-ESI+ chromatograms of some of the representative red and the yellow fungal color extracts. The top panel depicts chromatogram of the red fungal extract M1. (**A**) UV–vis chromatogram showing colored compounds only. (**B**) Total ion chromatogram (*m*/*z* 100–900) from positive ion electrospray. (**C**) UV–vis spectrum of PP-R. (**D**) Mass spectrum of PP-R from ESI+. The bottom panel depicts chromatogram of one of the yellow fungal extracts [V] from *Epicoccum nigrum* grown on YES media. (**E**) UV–vis chromatogram showing colored compounds only. (**F**) Total ion chromatogram (*m*/*z* 100–900) from positive ion electrospray. (**G**) UV–vis spectrum of orevactaene. (**H**) Mass spectrum of orevactaene from ESI+. The time delay between UV and MS signals is 0.05 min.

have also been shown by Juzlova et al. (*31*) for the ethanol extracts of *Monascus* pigments. This indicates that the red pigments produced by these *Penicillia* are either likely to be *Monascus* pigments or have similar chromophores as in *Monascus* pigments. Therefore, it is very likely that the red pigments produced by these *Penicillia* would also exhibit substitution reactions in their chromophores as shown by *Monascus* pigments. Hence, it should be possible to fermentatively produce newer hues in the red spectra by using different side chain precursors as in the case of *Monascus* pigment derivatives (*22*, *23*).

*Yellow Extracts.* The yellow extracts [S], [T], [U], [V], [W], and [W1] obtained from *E. nigrum* strains grown on one of the three media tested in this study showed relatively similar color

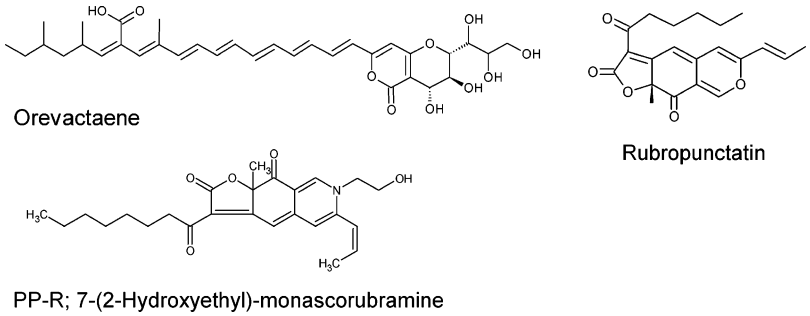


**Figure 5.** Characteristic structures of some fungal pigments detected and identified.

**Table 2.** LC-DAD Analysis Showing Pigment Composition of the Red and the Yellow Crude Fungal Extracts

| sample code[a] | total no. of major peaks[b] | retention time of major peaks[b] (min) | UV−vis absorption wavelength (nm) (within brackets, % of UV-max; in bold, VIS region) |
|---|---|---|---|
| M (red) | 1 | 7.88 | 210 (82), 250 (77), 278 (64), **422 (87)**, **518 (100)** |
| M1 (red) | 2 | 7.08 (peak 1) | 200 (35), 246 (65), 280 (63), **424 (83)**, **512 (99)** |
| | | 10.97[c] (peak 2) | 202 (100), 256 (90), **412 (55)**, **528 (41)** |
| N (red) | 4 | 7.85 (peak 1) | 200 (100), 260sh[f], 320sh[f], **424 (19)**, **518 (32)** |
| | | 8.48 (peak 2) | 204 (100), 282 (18), **424 (10)**, **524 (11)** |
| | | 8.91 (peak 3) | 204 (100), 272 (57), **430 (24)**, **518 (33)** |
| | | 12.19 (peak 4) | 202 (100), 250 (33), 278 (31), **430 (42)**, **518 (48)** |
| N1 (red) | 2 | 6.93 (peak 1) | 248 (58), 286 (25), **430 (70)**, **522 (100)** |
| | | 12.17 (peak 2) | 238 (69), 288 (27), **400sh**,[f] **462 (100)** |
| O (red) | 1 | 8.47 (peak 1) | 248 (38), 280 (52), **424 (92)**, **520 (100)** |
| P (red) | 2 | 13.09 (peak 1) | 224 (55), 298 (20), **468 (100)** |
| | | 13.41[d] (peak 2) | 216 (54), 246 (58), 288 (42), **460sh**,[f] **474 (100)** |
| Q (yellow) | 3 | 10.73 (peak 1) | 218 (100), 268 (45), **406 (43)** |
| | | 19.69 (peak 2) | 222 (100), 260sh[f], 268 (66), **404 (56)** |
| | | 20.39 (peak 3) | 218 (100), 268 (44), **404 (55)** |
| R (yellow) | 3 | 8.93 (peak 1) | 222 (58), 264 (28), 318 (100), 375sh,[f] **455 (8)** |
| | | 11.20 (peak 2) | 220 (63), 260 (48), 316 (100), **442 (21)** |
| | | 11.51 (peak 3) | 222 (82), 262 (58), 288sh,[f] 318sh,[f] 326 (100), **472 (18)** |
| S (yellow) | 2 | 11.64[e] (peak 1) | 200 (17), 280sh[f] 344 (20), **438 (100)** |
| | | 12.92 (peak 2) | 230 (20), 280sh[f] 342 (20), **440 (100)** |
| T (yellow) | 2 | 11.65[e] (peak 1) | 238 (17), 280sh[f] 344 (20), **440 (100)** |
| | | 12.93 (peak 2) | 218 (16), 280sh[f] **434 (100)** |
| U (yellow) | 2 | 11.63[e] (peak 1) | 238 (16), 280sh[f] 344 (20), **440 (100)** |
| | | 12.92 (peak 2) | 238 (16), 280sh[f] 344 (19), **436 (100)** |
| V (yellow) | 2 | 11.67[e] (peak 1) | 238 (16), 280sh[f] 344 (20), **438 (100)** |
| | | 12.92 (peak 2) | 232 (17), 280sh[f] 344 (20), **438 (100)** |
| W (yellow) | 2 | 11.64[e] (peak 1) | 236 (16), 280sh[f] 344 (20), **440 (100)** |
| | | 12.92 (peak 2) | 232 (15), 280sh[f] 338 (19), **440 (100)** |
| W1 (yellow) | 2 | 11.64[e] (peak 1) | 238 (17), 280sh[f] 344 (20), **440 (100)** |
| | | 12.93 (peak 2) | 200 (29), 238 (12), 280sh[f] 344 (39), **436 (100)** |

[a] As in **Table 1**. [b] Contributing ≥10% of peak area. [c] Identified as PP-R. [d] Identified as rubropunctatin. [e] Identified as orevactaene. [f] sh, shoulder.

compositions comprised of two predominant colored compounds. Therefore, these yellow extracts represented relatively similar hues in the yellow spectrum (**Figure 2**). One of those two compounds was identified to be the polyketide, orevactaene, as shown from one of these extracts (bottom panel of **Figure 4**). This oxopolyene compound was previously reported (*32*) to be produced also by *E. nigrum.* As compared to the extracts from *Epicoccum* cultures, the color composition of extracts [Q] and obtained from *P. atrovenetum* and *P. herquei* respectively, was markedly different, comprised of three major colored components each. The absorption spectra in the visible region of those three major colored components of extracts (Q) and (R) showed two different patterns comprised of absorption maxima at 404−406 and 442−472 nm, respectively, and hence resulting in a relatively different yellow hue than the Epicoccal extracts (**Table 2** and **Figure 2**).

In conclusion, we have shown that there is a great potential in the extraordinary color range of pigments produced by ascomycetous fungi in the red and the yellow spectra, although there are definitely more color shades that need to be explored from the other parts of the visible spectrum. *Monascus* pigments have been known for a long time, but there exists other pigment-producing genera, the colors of which resemble some of the commercially available colorants in terms of hue and chroma in the yellow spectra. Also, fungal colorants including *Monascus* colorants seem to provide additional hues in the red spectra. In addition, a multivariate approach based on the CIELAB color values seems to be a very useful tool in the colorimetric characterization of colorants. An examination of the stability, including pH and heat stability, of a few of the most promising fungal pigment extracts characterized in the present study are underway in our laboratory.

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# *Chapter 5*

*Computerized Screening for Novel Producers of Monascus-like Food pigments in Penicillium Species*

# Chapter 5: Computerized Screening for Novel Producers of *Monascus*-like Food Pigments in *Pencillium* Species

This chapter is based on the manuscript "Computerized Screening for Novel Producers of *Monascus*-like Food Pigments in *Pencillium* Species"

Sameer AS Mapari, Michael E. Hansen, Anne S. Meyer, and Ulf Thrane.

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## 5.1 Background

### 5.1.1 *Monascus* pigments: Polyketide pigments of the Orient

Pigments of *Monascus* spp. have been traditionally used as natural colorants in the Orient for centuries. *Monascus* pigments comprise of six main pigments *viz.* monascin and ankaflavin (yellow); monascorubrin and rubropunctatin (orange); and monascorubramine and rubropunctamine (purple- red). The same color exists in two molecular structures differing in the length of the aliphatic side chain. The yellow and the orange pigments are produced mainly in the cell-bound state and therefore have low water solubilities. However, the orange pigments have a unique azaphilone structure that imparts them a high affinity for compounds with primary amino groups (aminophiles such as amino acids, peptides, and proteins). Their reaction with primary amino group results in the water soluble counterparts. This property of such azaphilone pigments is very interesting as the resulting counterparts are not only more water soluble but also more light-stable and the color hue is more red than orange.

Despite the successful use of *Monascus* pigments in Asia, they are not allowed both in the European Union and the United States mainly due to the risk of coproduction of a known mycotoxin citrinin. This particularly draws an attention towards exploration of enormous fungal biodiversity for novel sources of such polyketide based azaphilone pigments as they are water soluble and provide a broader color range compared to carotenoids, and also carotenoid pigments to a large extent (relatively) are already commercially investigated. Moreover, *Monascus* pigments have been consumed over hundreds of years without an acute case of health hazard meaning that they must not be toxic if used in an appropriate dosage. Therefore, *Monascus* pigments and/or their derivatives were explored in a safer producer that would not coproduce any known mycotoxin particularly citrinin.

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# Computerized Screening for Novel Producers of *Monascus*-like Food Pigments in *Penicillium* Species

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*Monascus* pigments have been used as natural food colorants in Asia for centuries. They are not authorized for use in the European Union and the United States mainly due to the risk of coproduction of the mycotoxin citrinin by *Monascus* spp. In the present study, we screened for novel producers of *Monascus*-like pigments from ascomycetous filamentous fungi belonging to *Penicillium* subgenus *Biverticillium* that are not reported to produce citrinin or any other known mycotoxins. The screening was carried out using the X-hitting algorithm as a tool to quickly screen through chromatographic sample data files of 22 different *Penicillium* extracts with 12 *Monascus* pigment extracts as controls. The algorithm searched for the most similar UV−vis spectra of the metabolites (cross hits) present in the pigment extracts to those of the selected reference metabolites viz. monascin, rubropunctatin, rubropunctamine, and citrinin. The cross hits were then manually identified on the basis of their UV−vis and mass spectra. X-hitting was found to be a good tool in the rapid screening of crude pigment extracts. *Monascus* pigments were discovered in the extracts of two closely related species of *Penicillium* that were only distantly related to the genus *Monascus*. Monascorubrin, xanthomonasin A, and threonine derivatives of rubropunctatin were identified in the extract of *Penicillium aculeatum* IBT 14263, and monascorubrin was identified in the extract of *Penicillium pinophilum* IBT 13104. None of the tested *Penicillium* extracts showed the presence of citrinin. Thus, the present study brought out two novel promising sources of yellow, orange, and purple-red *Monascus*-like food pigments in the species of Penicillia that do not produce citrinin and opened the door to look for several more new promising sources of natural food colorants in the species of Penicillia.

**KEYWORDS: Computerized screening; *Monascus* pigments; polyketide; citrinin; X-hitting; *Penicillium***

## INTRODUCTION

The production of many currently authorized natural food colorants has a number of drawbacks, including a dependence on the supply of raw materials, which are influenced by agro-climatic conditions (*1*). Ascomycetous fungi provide an alternative source of naturally derived food colorants that could easily be produced in high yields because of the availability of cultivation technology and potential use of metabolic engineering tools. For centuries, *Monascus* spp. have been used for the production of red mold rice that has served as a source of a natural food colorant and/or spice in cooking in East Asia, particularly in Japan and China (*2, 3*). The red rice phenomenon is a result of the red pigment production by *Monascus* species; the six main *Monascus* pigments (*2, 3*) are ankaflavin, monascin, monascorubrin, rubropunctatin, rubropunctamine, and monascorubramine (compounds **1**−**6**, **Figure 1**). Other pigments like xanthomonasin A and B (*4, 5*), monankarines, (*6*) and industrially useful polyketide metabolites such as cholesterol-lowering compounds referred to as monacolins (*2, 7*) are also produced by the genus *Monascus*. *Monascus* pigments are originally cell bound and hydrophobic but contain an aminophilic moiety that reacts with amino group-containing compounds in the medium, such as proteins, amino acids, and nucleic acids, to form water-soluble pigments. In this direction, *Monascus* pigments derived with glutamic acid (*8*−*10*), aspartic acid, and alanine (*11*) have been identified and characterized. Jung et al. (*12*) fermentatively produced *Monascus* pigment derivatives using 20 individual amino acids as side chain precursors. The pigment derivatives obtained in this way were found to be more hydrophilic than their counterparts and more light-stable (*13*). Moreover, various red color hues were exhibited by these pigment derivatives, and the hues of most of these were darker red than their counterparts (*12, 13*). This makes these derivatives

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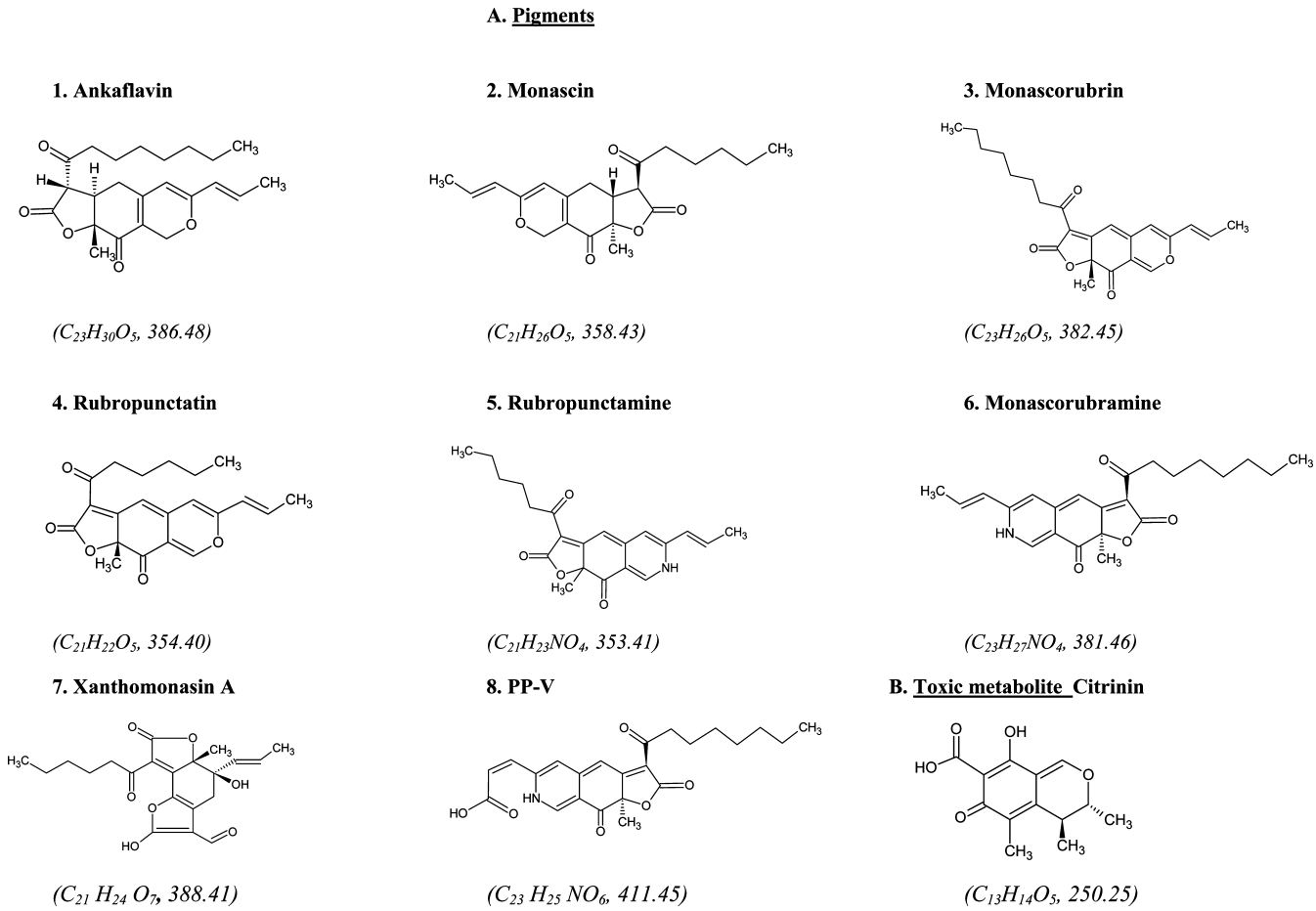


**Figure 1.** Structures of six characteristic pigments, a pigment, a pigment derivative, and a toxic metabolite of *Monascus* origin detected and identified in the present work (formula and nominal masses in parentheses) (1, 2, and 7, yellow; 3 and 4, orange; and 5, 6, and 8, purple-red).

quite interesting for further exploration as food colorants. However, *Monascus* spp., except a few commercial strains, wild types, and mutants, have also been shown to produce a mycotoxin called citrinin (**Figure 1**) on some media under certain conditions (*14, 15*). Even though both genetic attributes and culture conditions influence citrinin production, there remains a possibility of the presence of this mycotoxin in some batches, posing a critical safety issue in the pigment production process. Citrinin has been detected in some commercial red rice products, which are used as food colorants mainly in Asia (*14*). In addition to citrinin, which has been the major potential threat so far, other potential toxic metabolites of *Monascus* such as monascopyridines (*16*) and their toxic effects (*17, 18*) have also been reported from the red rice recently. This limits the food use of *Monascus* pigments, which are not permitted in Europe or in the United States, and puts a large incentive to screen for other fungi that produce *Monascus* pigments and/or *Monascus*-like pigments but no citrinin and/or other mycotoxins.

It is a well-known fact that the polyketide pathway, leading to the synthesis of the colored azaphilone compounds like *Monascus* pigments and their derivatives, is widely distributed in the fungal world. Also, a number of reports have been published on colored azaphilone compounds produced by fungi other than *Monascus* spp (*19*−*22*). Therefore, we hypothesized that *Monascus* or *Monascus*-like azaphilone pigments are likely to be produced by species in the *Penicillium* subgenus *Biverticillium* that produce copious amounts of red to orange-red pigments. It has also been reported that fungal polyketide-based metabolites like sclerotiorin, rotiorin, and *Monascus* pigments monascin and rubropunctatin form the same biogenic family, which might include such mycotoxins as citrinin (*23*). Therefore, the present work focuses on identifying *Monascus* or *Monascus*-like pigments in the species belonging to *Penicillium* subgenus *Biverticillium.* At the same time, the strains were analyzed for the absence of citrinin. For screening of fungi aimed at biotechnological production of pigments for food use, it is necessary to make sure that the pathogenic and/or toxigenic pigment-producing fungi are pre-eliminated at an early step. In the light of this, we aimed at intelligent screening (*24*), whereby we relied on a priori chemotaxonomic knowledge; there are several species in the *Penicillium* subgenus *Biverticillium* that produce red to orange-red pigments and are neither toxigenic nor pathogenic, and they have been used to preselect some isolates from our IBT Culture Collection at the Technical University of Denmark (DTU). Another effective tool of intelligent screening is a new method for the systematic and automated computer-assisted search of full UV spectra in a large number of data files for new natural products based on the new mathematical algorithm X-hitting (*25, 26*). In the present study, we used X-hitting as a tool to quickly screen through a large number of chromatographic sample data files and came out with the most likely candidates (cross hits) that have similar UV−vis spectra to those of the selected *Monascus* pigments and citrinin, used as the reference compounds. The cross hits were then manually identified on the basis of their UV−vis and mass spectra. To evaluate the citrinin-producing ability of *Monascus* strains on three different media under similar growth conditions to those of Penicillia, *Monascus ruber* and *Monascus purpureus* strains were used as controls.

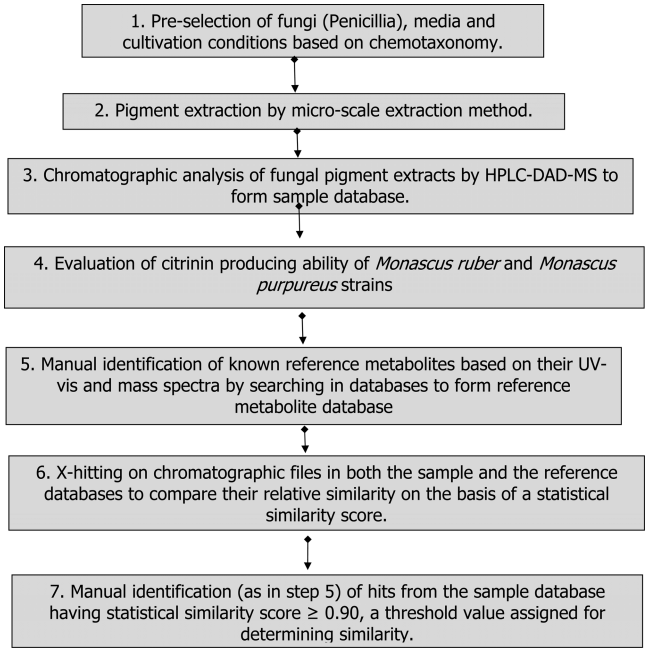


**Figure 2.** Schematic presentation of the method used in the present study.

## MATERIALS AND METHODS

A schematic presentation of the overall methodology used in the present study is shown in **Figure 2**. Specific steps and analytical techniques used in the methodology were as follows.

**Preselection of Fungi, Media, and Cultivation Conditions.** All fungal isolates used in this study were procured from the IBT Culture Collection at Center for Microbial Biotechnology, Technical University of Denmark (Kgs. Lyngby, Denmark). The fungal isolates were listed by the IBT numbers. All fungi were cultivated on either of the five different solid media, viz. yeast extract sucrose (YES) agar, malt extract agar (MEA), oatmeal (OAT) agar, potato dextrose (PD) agar, and Czapek−Dox yeast autolysate (CYA) agar (*27*), or in specific combinations on which maximum pigment was found to be produced with interesting color hues in the red to yellow spectra. The cultures were incubated in the dark at 25 °C for 7 days. **Table 1** represents the identity of the fungi, the IBT number, and the media used for their incubation.

**Extraction of Fungal Pigments.** Extraction was carried out by a modified version of the microextraction method (*28*), where 6 mm plugs were extracted in two steps in a 2 mL vial for 30 min, first using 1 mL of ethyl acetate with 0.5% formic acid to break open the cell wall and extract relatively apolar metabolites. The extract so obtained was then transferred to a new 2 mL vial and evaporated in vacuo. The second extraction was performed using 1 mL of isopropanol. By doing so, we could extract the maximum color. The second extract was then added to the vial with the residue from the previous extraction. It was then evaporated in a rotational vacuum concentrator (RVC; Christ Martin, Osterode, Germany). The residue was redissolved in 400 $\mu$L of methanol, in an ultrasonic bath (Branson 2510, Kell-Strom, Wethersfield, United States) for 10 min and filtered through a 0.45 $\mu$L PTFE syringe filter (SRI, Eatontown, NJ).

**Chromatographic Analysis.** High-resolution liquid chromatography−diode array detection−mass spectrometry (LC-DAD-MS) was performed on an Agilent HP 1100 LC system with a DAD and a 50 mm × 2 mm i.d., 3 $\mu$m, Luna C 18 II column (Phenomenex, Torrance, CA). The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, United Kingdom) with a Z-spray electrospray ionization (ESI) source and a LockSpray probe and controlled by the MassLynx 4.0 software. The MS system was operated in the positive ESI mode using a water−acetonitrile gradient system as described by Nielsen et al. (*29*).

**Analysis of LC-DAD-MS Data.** The presence of metabolites involved in the present study, (**Figure 1**) including both reference metabolites as well as cross hits and new hits was detected in $ESI^+$ from the first scan function of the reconstructed ion chromatograms. Standard compound citrinin (Sigma-Aldrich) was run with the pigment extracts under study, which was used as a reference for mass validation. The accuracy of mass detection by the instrument was in the acceptable range based on a calibration of the detected monoisotopic masses of the metabolites and the detected monoisotopic mass of standard citrinin. The UV−vis spectrum was obtained from the UV−vis chromatogram after background subtraction. The DAD-MS data for each of these compounds are shown below; however, we show LC-DAD-$ESI^+$-MS chromatograms of two reference metabolites and three new hits as the representative examples (**Figures 3** and **5**).

**Table 1.** Identity of the Analyzed Extracts with Their Respective X-Hit Numbers Used in the Present Study

| X-hit no. | fungal name | IBT no. | media |
|---|---|---|---|
| 1 | *Penicillium purpurogenun* | 11180 | CYA |
| 2 | | | YES |
| 3 | *P. pinophilum* | 3757 | PD |
| 4 | | | OAT |
| 5 | *P. pinophilum* | 14263 | CYA |
| 6 | | | YES |
| 7 | | | MEA |
| 8 | *P. pinophilum* | 13104 | PD |
| 9 | | | YES |
| 10 | | | OAT |
| 11 | *P. purpurogenum* | 21723 | YES |
| 12 | | | CYA |
| 13 | *P. funiculosum* | 3954 | MEA |
| 14 | | | CYA |
| 15 | *P. purpurogenum* | 3967 | CYA |
| 16 | *P. purpurogenum* | 21347 | PD |
| 17 | | | OAT |
| 18 | *P. purpurogenum* | 23082 | MEA |
| 19 | *P. aculeatum* | 14129 | CYA |
| 20 | *P. funiculosum* | 21276 | PD |
| 21 | *P. minioluteum* | 18368 | YES |
| 22 | *P. aculeatum* | 14259 | CYA |
| 23 | *Monascus ruber* | 98585 | MEA |
| 24 | *M. ruber* | 7904 | MEA |
| 25 | *M. ruber* | 9658 | YES |
| 26 | | | PD |
| 27 | *M. ruber* | 9655 | MEA |
| 28 | | | PD |
| 29 | *M. purpureus* | 9664 | PD |
| 30 | | | MEA |
| 31 | | | YES |
| 32 | *M. purpureus* | 9667 | PD |
| 33 | | | MEA |
| 34 | | | YES |
| 35 | citrinin standard | | |

*Ankaflavin.* Ankaflavin was detected as *m/z* 387.23 $[M + H]^+$ and confirmed by the adduct *m/z* 450.25 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was $\lambda_{max}$ 232, 283 (shorter peak), and 390.

*Monascin.* Monascin was detected as *m/z* 359.19 $[M + H]^+$ and confirmed by the adduct *m/z* 422.19 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was $\lambda_{max}$ 234, 292 (shorter peak), and 394.

*Rubropunctatin.* Rubropunctatin was detected as *m/z* 355.15 $[M + H]^+$ and confirmed by the adducts *m/z* 377.14 $[M + Na]^+$ and 418.16 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was $\lambda_{max}$ 246, 288, and 474.

*Rubropunctamine.* Rubropunctamine was detected as *m/z* 354.19 $[M + H]^+$ and confirmed by the adducts *m/z* 376.20 $[M + Na]^+$ and 417.20 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was $\lambda_{max}$ 306, 414, and 530 with a shoulder at 256.

*Citrinin.* Standard citrinin was detected as *m/z* 251.48 $[M + H]^+$ and confirmed by the adduct *m/z* 314.44 $[M + Na + CH_3CN]^+$ and the fragment 233.49 $[M + H - H_2O]^+$. The UV spectrum was $\lambda_{max}$ 216, 322 with a shoulder at 242.

*Monascorubrin.* Monascorubrin was detected as *m/z* 383.41 $[M + H]^+$ and confirmed by the adducts *m/z* 405.40 $[M + Na]^+$ and 446.36

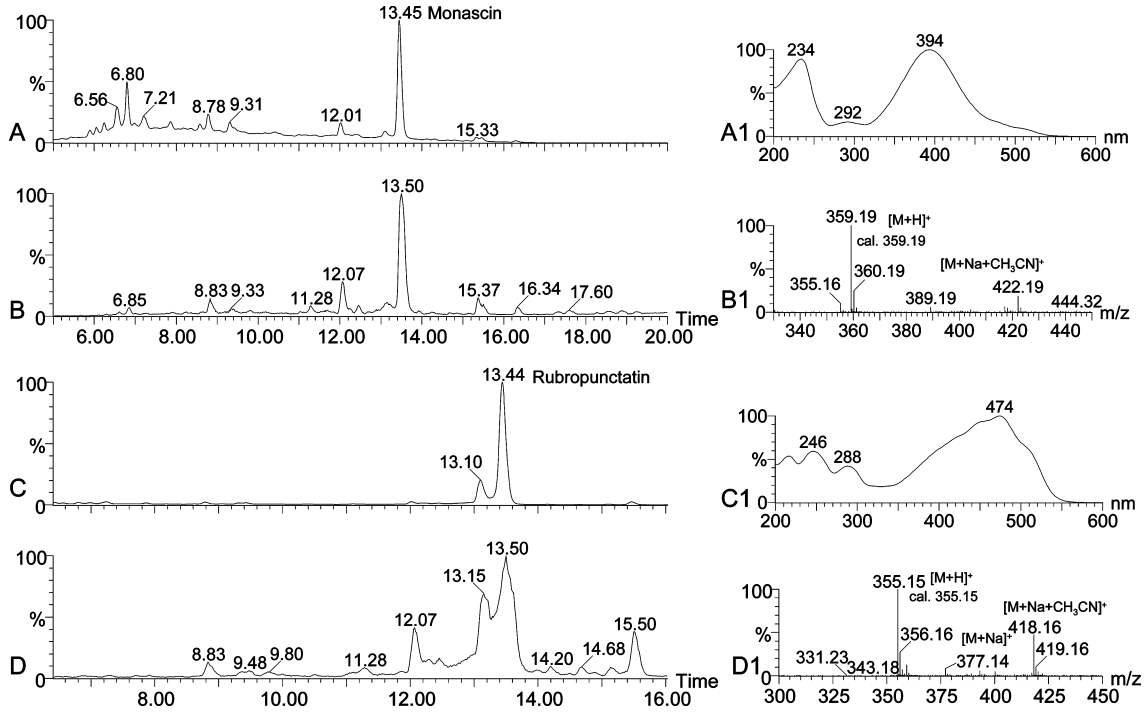


**Figure 3.** Top panel (**A** and **B**) depicts chromatograms showing monascin in the pigment extract of *Monascus ruber* IBT 9658 on PD (X-hit 26). (**A**) UV−vis chromatogram of 390−700 nm showing colored compounds only. **A1** represents the UV−vis spectrum of monascin. (**B**) Total ion chromatogram (*m*/*z* 100−900) from positive ion electrospray. **B1** represents the mass spectrum of monascin from $ESI^+$. Bottom panel (**C** and **D**) depicts chromatograms showing rubropunctatin in the pigment extract of *M. ruber* IBT 98585 on MEA medium (X-hit 23). (**C**) UV−vis chromatogram of 390−700 nm showing colored compounds only. **C1** represents the UV−vis spectrum of rubropunctatin. (**D**) Total ion chromatogram (*m*/*z* 100−900) from positive ion electrospray. **D1** represents the mass spectrum of rubropunctatin from $ESI^+$.

$[M + Na + CH_3CN]^+$ and the fragment 339.51 $[M + H - CO_2]^+$. The UV−vis spectrum was $\lambda_{max}$ 246, 286, and 478.

*Xanthomonasin A.* Xanthomonasin A was detected as *m*/*z* 389.44 $[M + H]^+$ and confirmed by the adduct *m*/*z* 452.39 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was $\lambda_{max}$ 234, 288 (shorter), and 394.

*PP-V.* PP-V was detected as *m*/*z* 412.21 $[M + H]^+$ and confirmed by the adducts *m*/*z* 434.20 $[M + Na]^+$ and 475.22 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was $\lambda_{max}$ 204, 300, 418, and 522 with a smaller peak at 252.

*Threonine Derivative of Rubropunctatin.* The threonine derivative of rubropunctatin was detected as *m*/*z* 456.36 $[M + H]^+$ and confirmed by the adducts *m*/*z* 478.31 $[M + Na]^+$ and 519.28 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was $\lambda_{max}$ 204, 282, 424, and 524.

Monascorubramine was not detected in the pigment extracts under study.

**X-Hitting.** *Selection of Reference Metabolites.* The fungus *Monascus* typically produces six major pigments (**Figure 1**): the yellow pigments ankaflavin and monascin, the orange pigments rubropunctatin and monascorubrin, and the purple-red pigments rubropunctamine and monascorubramine. We selected one of the pigments from each color viz. monascin (yellow), rubropunctatin (orange), and rubropunctamine (purple-red) that also represented the three different chromophores present in the azaphilone structures of six major *Monascus* pigments. This selection was based on the fact that the x-hitting would hit the UV−vis spectra of pigments having similar chromophores but different side chains almost equally well as the UV−vis spectra are relatively independent of the length of the side chain. The reference metabolites were manually identified in the three *Monascus* pigment extracts used as standards (X-hit 23, 24, and 26 in **Table 1**) and were used as reference metabolites. In addition to pigments, a standard of citrinin (Sigma-Aldrich) was also used as a reference metabolite.

*Metabolite and Sample Database and Their Interrelationship.* Two databases viz. metabolite and sample database, both of which were based on UV−vis spectra extracted from the DAD scan function of LC-MS analyses of standards and pigment extracts, were created. The metabolite database contained the information about the reference spectra from samples where the presence of known target metabolite was identified. In the present study, three pigments together with citrinin formed our reference metabolite database. The sample database contained information about the unknown spectra obtained from the analysis of the pigment extracts. The spectra of the reference metabolites were used as the fingerprint spectra, and a match factor was calculated and evaluated between the spectra at given retention times for each reference spectrum in the compound database (**Figure 2**). The spectra were evaluated based on a value describing its retention time (UV scan number). The interrelationship between the two databases was determined on the basis of this match factor, and a statistical similarity score was given.

*Matching Algorithm.* The algorithm behind X-hitting is described elsewhere (*25*).

## RESULTS AND DISCUSSION

**Identification of Metabolites Used in the Present Study.** *Reference Metabolites and/or Cross Hits.* Because commercial standards of reference metabolites were not available except for the standard citrinin, the reference metabolites used in the present study were detected and identified in the selected control *Monascus* pigment extracts (**Figure 3**). Identification was based on both the UV−vis spectra and the accurate masses; monoisotopic masses are given in the database such as Antibase (Wiley-VCH, Weinheim, Germany) as well as in the available literature references. The UV−vis and mass spectral data of the three reference metabolites viz. monascin, rubropunctatin, and rubropunctamine matched well with the data given by Teng et al. (*30*) and Su et al. (*31*). It must be noted that there are some discrepancies in the available literature with regards to identification of the six characteristic *Monascus* pigments on the basis of the UV−vis and mass spectra. For instance, Akihisa et al. (*5*) reported the spectra of the characteristic yellow pigments ankaflavin and monascin in the visible range to be $\lambda_{max}$ at 460 nm, and a $\lambda_{max}$ of 530 nm was shown for the orange pigments

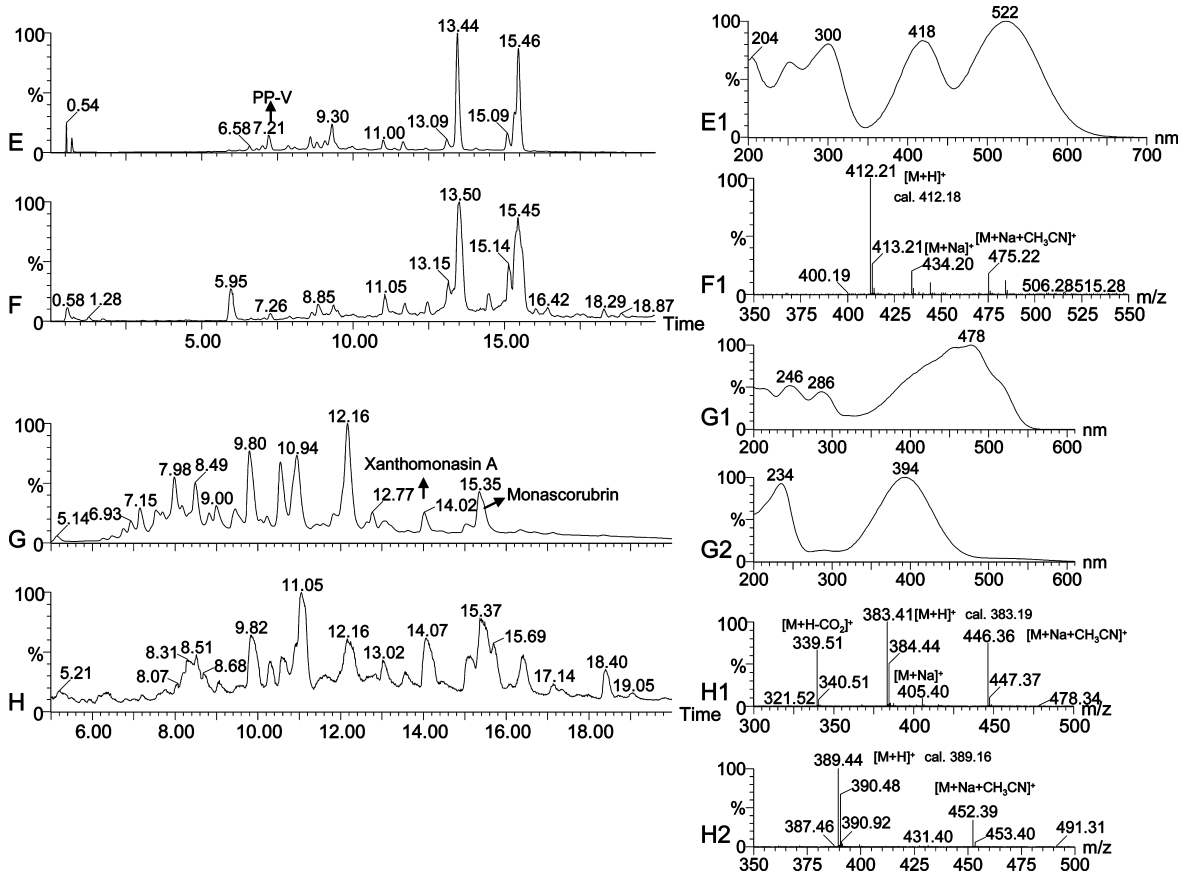


**Figure 4.** Top panel (**E** and **F**) depicts chromatograms showing new hit PP-V in the pigment extract of *Monascus purpureus* IBT 9664 on PD (X-hit 29). (**E**) UV−vis chromatogram of 390−700 nm showing colored compounds only. **E1** represents the UV−vis spectrum of PP-V. (**F**) Total ion chromatogram (*m*/*z* 100−900) from positive ion electrospray. **F1** represents the mass spectrum of PP-V from $ESI^+$. Bottom panel (**G** and **H**) depicts chromatograms showing new hits monascorubrin and xanthomonasin A in the pigment extract of *Penicillium aculeatum* IBT 14263 on YES medium (X-hit 5). (**G**) UV−vis chromatogram of 390−700 nm showing colored compounds only. **G1** represents the UV−vis spectrum of monascorubrin, and **G2** represents the UV−vis spectrum of xanthomonasin A. (**H**) Total ion chromatogram (*m*/*z* 100−900) from positive ion electrospray. **H1** represents the mass spectrum of monascorubrin, and **H2** represents the mass spectrum of xanthomonasin A from $ESI^+$.

monascorubrin and rubropunctatin. Moreover, the molecular weight for rubropunctatin was shown to be 353, and 355 − $[M + H]^+$ was shown for rubropunctamine. These results are neither in agreement with the previously reported (*30*−*33*) values nor with our own results. Thus, utmost care should be taken when comparing the UV−vis and/or mass spectral data to the data available in the literature for the identification of colored compounds, as this is an important step toward the discovery of new compounds.

*New Hits.* The identification of the new hits was carried out in the same way as for the reference metabolites and/or cross hits (**Figure 4**), and the data analyses are explained in the Materials and Methods section. The UV−vis and mass spectral data of ankaflavin matched well with the data given by Teng et al. (*30*) and Su et al. (*31*). The UV−vis and MS data for PP-V and threonine derivative of rubropunctatin were also well-supported by Ogihara et al. (*36*−*38*) and Jung et al. (*12*), respectively. The MS data for xanthomonasin A matched well with the data given by Akihisa et al. (*5*); however, the UV−vis spectrum for xanthomonasin A described by them is more toward orange ($\lambda_{max}$ 460nm) side of the visible spectrum. Our results indicated that the $\lambda_{max}$ was 394 nm, confirming its yellow nature.

**Citrinin-Producing Ability of the *Monascus* Cultures.** Before proceeding with the X-hitting experiment, the citrinin-producing ability of *Monascus* cultures was demonstrated under similar growth conditions to those of Penicillia in our laboratory on three different media. For this purpose, four *Monascus* cultures, two different strains from two preferably used species of *Monascus*, viz. *M. ruber* and *M. purpureus* [only the pigments of which are authorized for food use in Japan (*9*)], were selected. *M. ruber* IBT 9658 could produce citrinin (C+) only in YES media (**Figure 5A**) and *M. ruber* IBT 9655 did not produce any citrinin (C−) in the three tested media (**Figure 5B**), while *M. purpureus* cultures produced citrinin in all three tested media (**Figure 5C**,**D**). *M. purpureus* cultures produced both pigments and citrinin on YES, MEA, and PD media, while the pattern of citrinin and also pigments varied with respect to the media in case of *M. ruber* IBT 9658. IBT 9658 produced only pigments but no citrinin in PD and neither pigments nor citrinin in MEA and both citrinin and meager pigments in YES media (**Figure 5A**). *M. ruber* IBT 9655 in MEA produced pigments but no citrinin, and in PD medium, scanty pigmentation with no citrinin was found to be produced (**Figure 5B**). Thus, it can be inferred that the pigment and citrinin biosyntheses are independent of each other but the factors by which they are triggered are not clear in the case of the fungal strains belonging to the genus *Monascus.* This was also shown by Pisareva et al. (*34*) and Wang et al. (*15*). Even if the citrinin was not found to be produced by two strains on the media under consideration in the present study, it could very well be produced on other media like rice-based media, which is popularly used for the industrial production of these pigments. As it was shown in an important study (*35*) in China, where 35 *Monascus* strains used in food

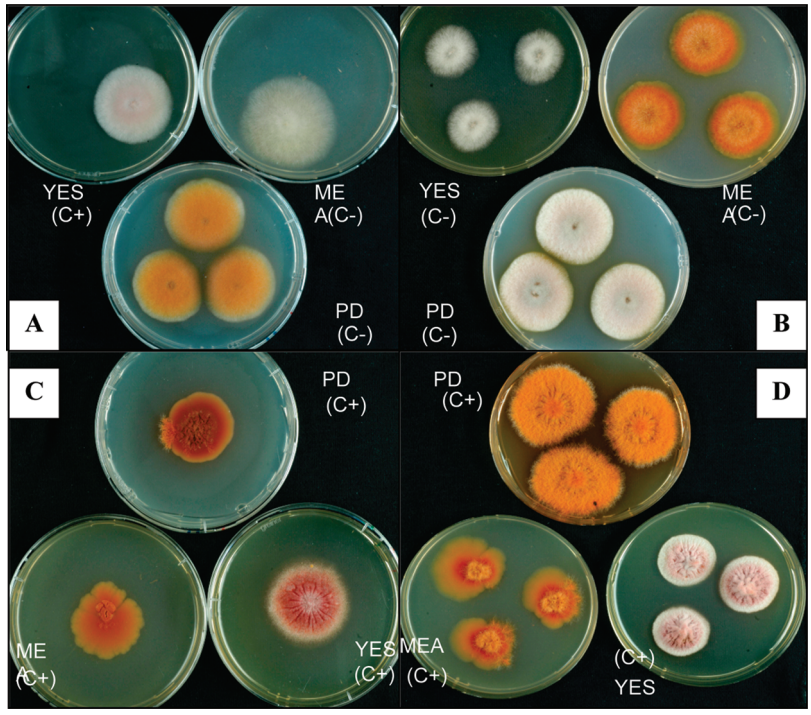


**Figure 5.** Citrinin and pigment-producing ability of *Monascus ruber* and *M. purpureus* strains in three different media. (**A**) *Monascus ruber* IBT 9658, (**B**) *M. ruber* IBT 9655, (**C**) *M. purpureus* IBT 9667, and (**D**) *M. purpureus* IBT 9664. MEA, PD, and YES represent the respective media, and C+ or C− in parentheses represent their ability to produce citrinin.

industry were selected to investigate the citrinin-producing ability in rice, all strains produced citrinin and thus posed a major safety issue. Another thorough investigation of 23 *Monascus* type cultures representing eight species for their citrinin-producing ability has revealed that citrinin was found to be produced in YES medium by all of them barring a couple of industrial strains (*15*). For the X-hitting experiment, extracts from pigment-producing citrinin positive or negative *Monascus* strains were used as positive or negative controls.

**X-Hitting.** Thirteen *Penicillium* strains from different species of subgenus *Biverticillium* grown on specific media that could elicit intense pigmentation were included in the present study. Some strains were also tested on two or more media for their ability to produce pigments in extractable amounts. Thus, 22 such combinations in addition to pigment-producing citrinin positive or negative *Monascus* strains (as mentioned earlier) were included, forming a total of 35 data files. These data files were given arbitrary numbers from one to 35 called as X-hit numbers (**Table 1**). Statistical similarity scores were obtained (as described in the Materials and Methods section Metabolite and Sample Database and Their Interrelationship) in gradation starting from the highest value (maximum 1) of that of the reference metabolite. A subjective threshold value of 0.900 of the statistical similarity score was set for all of the hits against each of the reference metabolites. Only those hits having a score value greater than or equal to the threshold value were manually identified in the LC-MS data files. It must be noted that the algorithm only uses the DAD scan function of the $ESI^+$ of LC-MS system as it is based on the UV−vis spectral similarity; however, in the future, we do plan to combine the two systems. The manual identification of hits resulted in cross hits and new hits (**Table 2**), which can be explained as follows: Cross hits were metabolites that had the maximum similarity (based on the statistical similarity score) of their UV−vis spectra to reference metabolites. Thus, they were the same as reference metabolites but were found in the sample data files other than reference data files. New hits were the other known metabolites that had very similar UV−vis spectra to those of the reference metabolites and were found either in the same or other files (than where reference metabolites were identified) at different retention times in the sample data files. The algorithm X-hitting enabled fast-paced (less than 15 min) and systematic identification of compounds in crude pigment extracts.

*Monascin Hits.* No cross hits were seen for monascin in the *Penicillium* extracts. All of the three cross hits (CH1, CH2, and CH3 in **Table 2**) for monascin were found to be present in the *Monascus* pigment extracts (controls): two from *M. purpureus* species and one from *M. ruber* species (**Tables 1** and **2**). The retention times of the cross hits were found to be almost identical to that of the reference monascin. Among the three new hits (NH1, NH2, and NH3 in **Table 2**), NH1 was found to be present in the *Penicillium* extract, while NH2 and NH3 were seen in the *Monascus* pigment extracts (controls). NH1 was identified by LC-DAD-MS analysis as xanthomonasin A produced by *Penicillium aculeatum* IBT 14263 on YES medium (**Figures 1** and **4**). NH2 and NH3 were identified as ankaflavin (LC-UV-$ESI^+$-MS chromatogram not shown) produced by *M. purpureus* IBT 9664 on YES medium and IBT 9667 on PD. The retention time for ankaflavin was 15.35 (**Table 2**), indicating its relative apolar nature due to the presence of a longer aliphatic chain ($C_7H_{15}$) as compared to monascin ($C_5H_{11}$) (**Figure 1**). The retention time for xanthomonasin A was slightly higher (**Table 2**) than monascin, indicating a relatively similar degree of polarity. The spectral similarity of these compounds can be explained by looking at their structures (**Figure 1**). The fact that ankaflavin, monascin, and xanthomonasin A—the three yellow pigments of *Monascus*—have similar chromophores (**Figure 1**) differing only in the side chains or aliphatic groups results in very similar spectra; hence, similarity scores of these compounds were found to be close to each other (**Table 2**).

*Rubropunctatin Hits.* No cross hits were seen for rubropunctatin in the *Penicillium* extracts. Four cross hits (CH4, CH5, CH5, and CH7 in **Table 2**) for rubropunctatin were found to be present in the *Monascus* pigment extracts (controls); two

**Table 2.** Discovery of Known Metabolites Based on the Spectral Similarity to Reference Metabolites by Manual Search in the LC-DAD-MS Data Files

| reference metabolites | retention time (min) | statistical similarity score (arbitrary with a maximum of 1.00) | cross hits (CH) or new hits (NH) or references (REF)/ retention time[a] | location of cross hits in terms of X-hit no. as per **Table 1** | identification[b] |
|---|---|---|---|---|---|
| monascin | 13.45 | 0.995 | REF/13.45 | 26 | monascin |
| | | 0.978 | CH1/13.46 | 32 | monascin |
| | | 0.969 | CH2/13.46 | 28 | monascin |
| | | 0.946 | CH3/13.45 | 31 | monascin |
| | | 0.965 | NH1/14.02 | 6[c] | xanthomonasin A |
| | | 0.951 | NH2/15.33 | 31 | ankaflavin |
| | | 0.973 | NH3/15.35 | 32 | ankaflavin |
| rubropunctatin | 13.44 | 0.989 | REF/13.44 | 23 | rubropunctatin |
| | | 0.965 | CH4/13.44 | 27 | rubropunctatin |
| | | 0.958 | CH5/13.43 | 24 | rubropunctatin |
| | | 0.955 | CH6/13.44 | 30 | rubropunctatin |
| | | 0.923 | CH7/13.44 | 33 | rubropunctatin |
| | | 0.952 | NH4/15.35 | 6[c] | monascorubrin |
| | | 0.936 | NH5/15.35 | 9[c] | monascorubrin |
| | | 0.960 | NH6/15.45 | 29 | monascorubrin |
| | | 0.950 | NH7/15.47 | 30 | monascorubrin |
| | | 0.952 | NH8/15.46 | 33 | monascorubrin |
| rubropunctamine | 9.42 | 0.975 | REF/9.42 | 24 | rubropunctamine |
| | | 0.960 | CH8/9.47 | 27 | rubropunctamine |
| | | 0.952 | CH9/9.47 | 29 | rubropunctamine |
| | | 0.943 | NH9/8.48 | 5[c] | threonine derivative of rubropunctatin |
| | | 0.958 | NH10/7.24 | 27 | PP-V |
| | | 0.950 | NH11/7.21 | 29 | PP-V |
| | | 0.966 | NH12/7.26 | 24 | PP-V |
| citrinin | 5.83 | 0.945 | REF/5.83 | 35 | citrinin |
| | | 0.938 | CH10/5.92 | 25 | citrinin |
| | | 0.937 | CH11/5.92 | 29 | citrinin |
| | | 0.929 | CH12/5.77 | 30 | citrinin |
| | | 0.934 | CH13/5.90 | 32 | citrinin |

[a] Cross hits were where metabolites were the same as reference metabolites that had the maximum similarity (based on the statistical similarity score) of their UV−vis spectra to reference metabolites and were found in the sample data files other than reference data files. New hits were where the other known metabolites that had very similar UV−vis spectra to those of the reference metabolites and were found either in the same or other files (than where reference metabolites were identified) at different retention times. [b] Identified based on UV−vis and mass spectra as shown in **Figures 3A**,**B** and **5A**,**B**. [c] Indicate their presence in the *Penicillium* extracts.

each from *M. ruber* and *M. purpureus* species, quite interestingly in the same medium MEA with a retention time of 13.43 (**Tables 1** and **2**). Among the five new hits (NH4, NH5, NH6, NH7, and NH8 in **Table 2**), NH4 and NH5 were found to be present in the *Penicillium* extract, while NH6, NH7, and NH8 were seen in the *Monascus* pigment extracts (controls). All of the new hits were identified by LC-DAD-MS analyses as monascorubrin (**Figures 1** and **4**). NH4 and NH5 were produced by *Penicillium* spp.; NH4 was produced by *P. aculeatum* IBT 14263 on YES medium, and NH5 was produced by *P. pinophilum* IBT 13104 on YES medium (**Tables 1** and **2**). NH6, NH7, and NH8 were found to be produced by *Monascus* strains, out of which NH6 and NH7 were found to be produced by *M. purpureus* IBT 9664 on PD and MEA and NH8 by *M. purpureus* IBT 9667 on MEA (**Tables 1** and **2**). The retention time of monascorubrin ranged from 15.35 to 15.47 (**Table 2**). A slight difference in the retention time could be due to the day-to-day variation in the chromatographic analyses as they were performed on two different days using solvents of different batch and purity. A relatively higher retention time of monascorubrin than rubropunctatin can be explained in terms of its higher hydrophobicity because of the presence of longer aliphatic chains as in case of ankaflavin and monascin (**Figure 1**). The spectral similarity of the two compounds also lies in their similar chromophores just like in the case of ankaflavin and monascin as explained earlier.

*Rubropunctamine Hits.* No cross hits were seen for rubropunctamine in the *Penicillium* extracts. Two cross hits (CH8 and CH9 in **Table 2**) for rubropunctamine were found to be present in the *Monascus* pigment extracts (controls), one each from *M. ruber* and *M. purpureus* species, respectively, with a retention time of 9.47, indicating the more polar water-soluble nature of this pigment (**Tables 1** and **2**). Among the four new hits (NH9, NH10, NH11, and NH12 in **Table 2**), NH9 was identified in the *Penicillium* extract as a threonine derivative of rubropunctatin (**Figure 1**), and it was found to be produced by *P. aculeatum* IBT 14263 on CYA medium. NH10, NH11, and NH12 were identified in the *Monascus* pigment extracts (controls) by LC-DAD-MS analyses as PP-V, 3-(9a-methyl-3-octanoyl-2,9-dioxo-2,7,9,9a-tetrahydro-furo[3,2-g]isoquinolin-6-yl) acrylic acid (**Figures 1** and **4**), a homologue of monascorubramine. NH10 was identified in the pigment extract of *M. ruber* IBT 9655 on MEA, NH11 in the pigment extract of *M. purpureus* IBT 9664 on PD, and NH12 in the pigment extract of *M. ruber* IBT 7904 on MEA (**Tables 1** and **2**). PP-V, a water-soluble purple pigment, was previously reported to be produced by an unidentified species of *Penicillium* (*36*−*38*) but, to the best of our knowledge, never in *Monascus* spp. As PP-V is a homologue of monascorubramine, the spectral similarity of the two compounds is self-explanatory. In a study by Jung et al. (*12*), glycine derivatives of *Monascus* pigments with side chains of $C_5H_{11}$ and $C_7H_{15}$ were formed, and the structure was analyzed

by $^{1}H$ NMR and $^{13}C$ NMR. A careful examination of the structure has shown that it has a similar structure as PP-V. The molecular formula of the glycine derivative of *Monascus* pigment with side chain of $C_5H_{11}$ was given as $C_{23}H_{25}NO_6$, which is the same as of PP-V (*36*). The structures of the other amino acid derivatives were thought to be similar to this derivative except for the amino acid moiety. This could very well explain the spectral similarity of threonine derivative of rubropunctatin to either PP-V or rubropunctatamine or monascorubramine, the amine form of rubropunctatin and monascorubrin, respectively. Sato et al. (*11*) also reported alanine and aspartate derivatives in commercial *Monascus* pigments, but this is the first ever report where an amino acid derivative of *Monascus* pigment was found in the pigment extract of *Penicillium* spp.

The earlier work (*39*) carried out on the chemistry of *Monascus* pigments suggests that the orange pigments viz. monascorubrin and rubropunctatin form the red pigments as the yellow pigments are unable to react with NH groups to produce the corresponding amines as seen from their structures (**Figure 1**). Our results indicated that the cross hit CH8 (rubropunctamine) and new hits NH9 (threonine derivative of rubropunctatin), NH11, and NH12 (PP-V) were found in the data files viz. X-hits 27, 5, 29, and 24, respectively (**Tables 1** and **2**). In these data files, except for the X-hit 5, either rubropunctatin or monascorubrin was also detected. This can be explained as the monascorubrin and/or rubropunctatin produced could react with either the free amino acids, as in case of MEA medium, or the amino acids of the mycelial pool in case of PD medium. As only high pH favors such reactions (*39*) and the drop in the pH of both the media at the time of pigment production, only part of monascorubrin or rubropunctatin could react to form substantially low amounts of amine forms such as rubropunctamine or PP-V or the threonine derivative of rubropunctatin (**Figures 1** and **4**).

*Citrinin Hits.* Four cross hits for citrinin (CH10, CH11, CH12, and CH13 in **Table 2**) were found to be present in the *Monascus* pigment extracts: CH10 from *M. ruber* IBT 9658 on YES medium, CH11, CH12, and CH13 from *M. purpureus* IBT 9664 on PD and MEA, and *M. purpureus* IBT 9667 on PD, respectively. The retention time of citrinin ranged from 5.77 to 5.92 (**Table 2**). The variation found is explained earlier. The citrinin negative controls of *Monascus* pigment extracts were shown as negatives by the algorithm, meaning that the similarity score was quite low for such hits. However, some false negatives were encountered. The reason could be the very low concentration of citrinin in these samples. It is likely that it was below the detectable range of DAD or that a very, very tiny peak was obtained such that its spectra went undetected by the algorithm or considered as noise. In such a case, they could only be detected manually by the mass and UV−vis spectra. Currently, we are working on such issues as this to make the algorithm more robust. Very interestingly, none of the pigment extracts from *Penicillium* cultures were found to contain citrinin by the algorithm. To be sure, a citrinin search was carried out manually in all of the *Penicillium* extracts, and all of them were found to be citrinin negative. This is quite significant as the production of citrinin, the major toxic metabolite of *Monascus*, prevents its food use by the authority both in the United States and the European Union.

**New Hits and Their Sources.** The fungus *P. aculeatum* IBT 14263 was found to produce two *Monascus* pigments viz. xanthomonasin A (yellow) and monascorubrin (orange) and one pigment derivative (purple-red). The fungus *Penicillium pinophilum* IBT 13104 was found to produce the orange *Monascus* pigment monascorubrin. It must also be mentioned that several unknown (not found in the databases used in the present study) metabolites in the yellow-orange-red spectrum were found in the extracts (data not shown) of other Penicillia (**Table 1**) used in this study. They are most likely to be *Monascus*-like polyketide-based azaphilone pigments, but it needs to be further investigated. *P. aculeatum* and *P. pinophilum* are closely related species; however, they are remotely related to the genus *Monascus*. The presence of polyketide-based *Monascus* pigments in these two fungi without the coproduction of citrinin forms an interesting avenue to be further explored. This is a significant discovery toward biotechnological production of naturally derived fungal food pigments as these are not known to produce any other known mycotoxins when grown in the media and under the laboratory conditions used in this study. Notably, the mycotoxins rubratoxin A and B have been reported to be produced by *P. rubrum* and *P. purpurogenum*, but this is not correct as these mycotoxins are produced by isolates of the species *P. crateriforme* (*40*). Fungus such as *Penicillium marneffei* belongs to the same group and produces copious amounts of red pigment, but it was deselected as it produces a known mycotoxin secalonic acid D and is also a well-known human pathogen as we have reported previously (*1*). This signifies the chemotaxonomic aspect of our screening methodology. We have also shown in a previous article that the color of some of these pigments including *Monascus* pigments provides additional hues in the red spectra, not covered by some of the commercially available colorants (*41*). PP-V was earlier reported to be produced by *Penicillium* spp. but never in *Monascus* spp. Our results have shown it to be produced by *M. ruber* IBT 7904 and 9655 and *M. purpureus* IBT 9664.

In conclusion, we have shown two newer promising sources of red to orange-red natural food colorants in the species of *Penicillium* that do not produce citrinin, with a possibility of finding several more from this group of fungi. These Penicillia are not reported to produce any other known mycotoxins. In addition, X-hitting was found to be good tool in the rapid screening of crude pigment extracts. Further necessary testing of algorithm is in progress to improve its application as a screening tool, and the pigments are being evaluated for their functionality.

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# *Chapter 6*

## *Photostability of Natural Orange-red and Yellow Fungal Pigments in Liquid Food model Systems*

# Chapter 6: Photostability of Natural Orange-red and Yellow Fungal Pigments in Liquid Food Model Systems

This chapter is based on the manuscript "Photo Stability of Natural Orange-red and Yellow Fungal Pigments in Liquid Food Model Systems"

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## 6.1 Background

Light stability is a key issue and the poor light stability is considered to be a bottle neck for the acceptance of *Monascus* pigments by the food industry. However, in the literature, there is no clear guidance on the acceptance threshold of the light stability data for the food applications. Hence, the interpretations may be biased or not taken into account the conditions of the storage light and/or the intensity used in the experiments conducted. Also, there is bound to be a variation in the light stability of natural pigments. The present study was undertaken to address this issue.

# Photostability of Natural Orange-red and Yellow Fungal Pigments in Liquid Food Model Systems

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ABSTRACT.

The variation in the photostability among the currently authorized natural pigments limits their application span to a certain type of food system and more robust alternatives are being sought after to overcome this problem. In the present study, the photostability of an orange-red and a yellow fungal pigment extract produced by ascomycetous fungi belonging to the genera *Penicillium* and *Epicoccum*, respectively, were studied in a soft drink model medium and in citrate buffer at low and neutral pH. The quantitative and qualitative color change pattern of the fungal pigment extracts indicated an enhanced photostability of fungal pigment extracts compared to the commercially available natural colorants *Monascus* Red and turmeric used as controls. Yellow components of the orange-red fungal pigment extract were more photostable than the red components. Chemistry of photodegradation of orange-red pigment extract was studied by HPLC-DAD-MS and a light induced formation of a structural analogue of sequoiamonascin C, a *Monascus*-like polyketide pigment discovered in the extract of *Penicillium aculeatum* IBT 14263 on YES medium, was confirmed in the soft drink medium at pH 7.

KEYWORDS: *Monascus* pigments, polyketide, *Epicoccum*, *Penicillium,* sequoiamonascin C.

BRIEFS Photostability of natural fungal pigment extracts

## INTRODUCTION

Naturally derived colorants look set to overtake synthetic food colorants in market value due to the rising demand for the clean label ingredients. The stability of natural colorants is quite an issue since on the one hand, the color stability is a prerequisite for successful application, while on the other hand, in order to absorb light and elicit a color visible to the human eye, most of the currently employed natural pigments are highly unsaturated compounds and are thus prone to light, heat, and oxygen disintegration and may change color depending on the pH (*1*). However, there are large differences in the stability of different classes of existing natural food colorants (*2*). Vegetable carbon and caramel are very stable towards heat, light, and oxygen. Carminic acid and carmine are also quite stable but not as stable as vegetable carbon and caramel. On the other hand, turmeric is rapidly bleached by light and beet root pigments turn brownish even under mild heating conditions (*2*). In many cases the stability issues in relation to heat, light, and pH currently limit the application span of certain natural colorants to certain types of products that "fit" the stability requirements of the colorant. Hence, there is a great scope for the discovery of relatively more stable natural pigments that may have wider industrial applications without the potential use of formulation techniques. Recently, microorganisms including ascomycetous fungi have attracted attention as additional or alternative readily available sources of naturally derived food colorants that are independent of agro-climatic conditions (*1, 3, 4*). Pigments produced by such kind of fungi can be broadly classified as carotenoids and/or structurally diverse polyketide class of pigments synthesized as secondary metabolites, the example of the latter kind are *Monascus* pigments that have been used as natural food colorants over a century in the East Asia (*3, 4*). However, *Monascus* spp., except a few commercial strains, wild types and mutants, have also been shown to coproduce a mycotoxin, citrinin (**Figure 1**) on some media under certain conditions (*5, 6*). This is one of the prime reasons for the ban on the use of *Monascus* pigments in foods in the European Union and in the United States. Recent studies have brought out the potential of pigment producing genera, other than *Monascus,* that have been chemotaxonomically selected over those which are either known human pathogens or produce

known mycotoxins (*1, 7-9*). The color hues of such pigment producing fungi have been shown to resemble and/or complement the color palette of contemporary natural food colorants in the red and the yellow spectra (*7*). Polyketide pigments such as *Monascus* pigments are known to be unstable towards UV and the visible light compared to their amino acid derivatives (*10*). Also not much is known about the chemistry behind the color change of fungal polyketide pigments including *Monascus* pigments in food systems that are exposed to light. Therefore, this present study was undertaken with the purpose of studying the light stability of two promising fungal pigment extracts characterized in our previous study (*7*). Natural colorants including fungal polyketide pigments are (in most cases) a mixture of several components and therefore it would be interesting to see the extent of stability of individual components towards light. We hypothesized that some of the components could be more photostable than the others and that their discriminative destabilization would affect the resultant color before and after the light exposure. In the light of this, the quantitative and qualitative color change pattern of an orange-red and a yellow pigment extract produced by ascomycetous fungi belonging to the genera *Penicillium* and *Epicoccum,* respectively, were studied. We used an application based approach of food colorants, which is based on the CIELAB color space, where by a beverage based medium, soft drink medium typically used by the food colorant industry, was chosen as one of the food systems, in which the effect of pH could also be studied. Citrate buffer was used as another food system parallel to the soft drink medium as citrate is a commonly used preservative in many food products. The pigment composition in each of the two food model systems before and after the light exposure was analyzed by high-resolution liquid chromatography-diode array detection-mass spectrometry (LC-DAD-MS). The prime objective was to compare the photostability of the fungal pigment extracts to turmeric and a commercially available *Monascus* Red colorant used as controls for the yellow and the orange-red colorants in our study.

## MATERIALS AND METHODS

**Pre-selection of fungi, media, cultivation conditions and food system composition.** Fungal isolates used in this study were procured from the IBT Culture Collection at Center for Microbial Biotechnology, Technical University of Denmark, Kgs. Lyngby, Denmark. The fungal isolates were listed by the IBT numbers. *Penicillium aculeatum* IBT 14263 was cultivated on Yeast extract sucrose (YES) agar, and *Epicoccum nigrum* IBT 41028 was cultivated on Potato dextrose (PD) agar (*11*). The cultures were incubated in the dark at 25 $^{o}$C for 7 days. Concentrated soft drink medium was kindly provided by Chr Hansen A/S (Hørsholm, Denmark). The concentrated medium contained (g/L): Sucrose for soft drink (EU category 1, refined sugar): 430; sodium benzoate, food grade: 0.7; potassium sorbate, food grade: 0.9; citric acid, food grade: 8.6; demineralized water: 559.8. This concentrated medium was then diluted 1:5 by demineralized water to get the working soft drink medium (SD). The resulting pH was 3.0 ± 0.2 with a °Brix level of 11.0 ± 1.0. The pH was changed using 0.1 M NaOH as per the need of the experiment. The model system of 0.1 M citrate buffer (CB) was prepared by mixing appropriate quantities of citric acid solution and sodium citrate solution until a desired pH was obtained.

**Control colorants**. Water soluble turmeric (Product code: T-PT8-WS) was kindly provided by Chr Hansen A/S (Hørsholm, Denmark) and *Monascus* Red colorant, designated as *Monascus* Red 3 (MR3), was a kind gift from Riken Vitamin Co. Ltd. (Japan).

**Extraction of fungal pigments.** Extraction was carried out by a modified version of the micro-extraction method (*12*): Larger plugs of 2.1 cm diameter were taken out from fungal mycelia and the agar surface of the agar culture where the pigment was diffused. The plugs were quickly frozen using liquid nitrogen and transferred to a freezer (-18 $^{o}$C) prior to their subsequent freeze drying at -60 $^{o}$C (Heto Drywinner, Holm and Halby, Denmark). The dried mass so obtained was crushed in pestle and mortar and a coarse powder was obtained, which was stored in a freezer in closely sealed polythene bags. This powder was used for the extraction of pigments. One g of fungal powder (from each species) was transferred to a vial and 20 mL of 96% of ethanol was added. The vials were kept in an ultrasonic bath (Branson 2510, Kell-Strom, Wethersfield, USA)

for 45 minutes. The extracted pigment was separated by filtration using Whatman filter paper no. 1. The fungal residue was transferred back into the vial and the extraction step was repeated until no more pigment could be extracted with lesser volumes of solvent (10 mL and subsequently with 5 mL). The crude pigment extract was collected in a round bottom flask and dried in a rotary evaporator (Büchi Rotavapor R-134, Flawil, Switzerland) at 32 °C and with a pressure at the beginning 300 mbar and gradually reducing it to 100 mbar. Since there should be no air in the flask, the vacuum in the flask with dried pigment residue was replaced with a stream of nitrogen to avoid air oxidation. The flask was closed tightly with a stopper and wrapped with a silver foil and preserved in the freezer for subsequent use. For experiments the dried pigment residue was re-dissolved in aqueous ethanol until it was completely soluble. This was then used as stock for adding colorants to the food system.

**Photostability study.** The colorants were added to 80 g of the two food systems until a desired lightness level (L-value) was obtained. The L-value was predetermined and kept in a range (see values range below) typically used by Chr. Hansen A/S for the soft drink medium based on their application experience. The L-value was measured using transmittance based chromameter (Minolta CT 310, Konica Minolta, Mahwah, NJ). The initial lightness values used for the colorants were as follows: Control *Monascus* Red 3 (MR3): 76.43±0.50; pigment extract from *Penicillium aculeatum* IBT 14263 on YES: 75.36±0.24; Turmeric: 97.67±0.21; pigment extract from *Epicoccum nigrum* IBT 41028 on PD: 97.8±0.29. The sample solutions were transferred to canted neck flasks (IWAKI Scitech div., Chiba, Japan, Asahi techno glass, 25 $cm^2$ / canted neck) filled up to the brim to prevent oxidation. The samples were put in a light cabinet (Suntest XLS+, Atlas-mts, Chicago, US, Xenon lamp, light intensity/h 172.8 $J/cm^2$, cut-on wavelength 250 nm) at 25 $^0$C, the position of the flasks noted (only 12 flasks could be fitted at a time). The light cabinet was programmed to run for a definite period of time after which the colorimetric values, L*, a*, b* of aliquots from samples were noted using the chromameter. The process was continued until maximal decoloration. Also, the locations of the flasks in the light cabinet were interchanged in order to ensure a uniform exposure of light per sample.

**Solid phase extraction**. Aliquots were withdrawn from the samples before and after the light exposure and solid phase extraction was performed as per our standardized method (*12*) with a slight modification in the washing step. Strata-X-C 33 µm cation mixed mode polymer columns (Phenomenex ApS, Allerød, Denmark) with a concentration of 60 mg/3 mL were used. One mL of methanol was used for conditioning followed by 1 mL of distilled water for calibration. Samples, acidified with 0.1% phosphoric acid, were loaded and washed with 0.1% phosphoric acid and de-ionized water for citrate buffer and soft drink medium respectively. Neutral and acidic components were eluted with methanol and alkaline components were eluted with 5% (v/v) ammonia in methanol. The alkaline elute was added acetonitrile with 50 ppm trifluoroacetic acid in 1:5 ratio prior to chromatographic analysis considering the pH instability of Luna C 18 II column (Phenomenex ApS, Allerød, Denmark) at alkaline pH.

**Chromatographic Analysis.** High-resolution LC-DAD-MS was performed on an Agilent HP 1100 liquid chromatograph (LC) system with a photodiode array detector (DAD) and a 50 × 2 mm i.d., 3 µm, Luna C 18 II column (Phenomenex ApS, Allerød, Denmark). The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, U.K.) with a Z-spray electrospray ionization (ESI) source, and a LockSpray probe and controlled by the MassLynx 4.0 software. MS system was operated in the positive ESI mode using a water-acetonitrile gradient system as described by Nielsen *et al.* (*13*).

**Analysis of LC-DAD-MS Data.** The colored components in the pigment extract were detected in the UV-vis chromatogram of 390-700 nm. The identification of sequoiamonascin C was based on both UV-vis and mass spectra from total ion chromatogram (m/z 100-900) from positive ion electro spray. The UV-vis spectrum was obtained after background subtraction. The DAD-MS data for sequoiamonascin C and its proposed analogue is shown below:

Sequoiamonascin C was detected as *m/z* 419.20 $[M + H]^+$ and confirmed by the adduct *m/z* 482.22 $[M + Na + CH_3CN]^+$. The UV-vis spectrum was $\lambda_{max}$: 232, and 390. The proposed structural analogue was detected as *m/z* 419.21 $[M + H]^+$ and confirmed by the adduct *m/z*

436.19 $[M + NH_4]^+$, and 482.21 $[M + Na + CH_3CN]^+$. The UV-vis spectrum was $\lambda_{max}$: 244, and 398.

RESULTS AND DISCUSSION

The transmittances of each of the fungal extracts and the reference colorants were adjusted to a particular L*-value in order to provide for a fair comparison of colorants with respect to photostability. The zero hour L-value range was decided based on the optimal range of existing natural colorants in the soft drink medium that gave a desirable hue. For instance, the color of the orange-red pigment extract was adjusted to a L*-value of 75-77, and for the yellow pigment extract it was in the range of 97-98. The color change was quantified by using ΔE* values which is the difference in the color from a reference point in the three dimensional CIELAB color space, where lightness is also considered along with colorimetric co-ordinates a* (red-green parameter) and b* (yellow-blue parameter) as seen from its formula given as:

$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$, where ΔL* is change in the lightness value with respect to time, Δa* is change in the red-green CIELAB coordinate a* with respect to time, and Δb* is change in the yellow-blue CIELAB coordinate b* with respect to time. The reference point for each individual sample was the color recorded at time 0 of the light exposure.

**Quantitative and qualitative color change of orange-red fungal pigment extract with MR 3 as control under light in soft drink medium and citrate buffer at two different pH.** The color change of fungal pigment extract was relatively more rapid at pH 5 than at pH 7 in both of the liquid food model systems, the soft drink medium (SD) being slightly better than the citrate buffer (CB) (**Figure 2**). The data for control for both CB and SD at pH 5 could not be recorded as it showed signs of degradation at 0 hour itself. The relatively slower decoloration of fungal pigment extract observed in the soft drink medium might be due to stabilizing effects by some unknown mechanism conferred by the other ingredients in the soft drink medium, e.g. sucrose, sodium benzoate or potassium sorbate. Also, the magnitude of decoloration was more in the first 2 hours for the two systems especially noticeable at pH 5 (**Figure 2**) indicating a classical first

order decay mechanism underlying the color change. It has been recently shown that the pigment extract obtained from *Penicillium aculeatum* IBT 14263 on YES medium had *Monascus* pigments and their derivatives such as monascorubrin (orange), xanthomonasin A (yellow), and threonine derivative of rubropunctatin (purple-red) (*9*). This might explain the relatively higher light stability of these pigment extracts at pH 7 as *Monascus* pigments in solution are more stable at near neutral and/or alkaline pH (*14, 15*). Compared to the control *Monascus* red colorant MR3 at pH 7, the color change of the orange-red pigment extract in both the food systems at pH 7 was less pronounced especially after 4 hours (**Figure 2**). This could be due to the fact that orange-red pigment extracts had relatively more yellow components than the control *Monascus* red colorant MR3. As it is previously reported that the red pigments of *Monascus* are less stable than the yellow pigments in solution (*14*), the continual decoloration of the control *Monascus* red colorant MR3 at pH 7 in the two tested food systems can be explained. Thus, it can be inferred that the relatively larger proportion of the yellow components in the orange-red pigment extract was the factor that determined its photostability in the two tested food systems at pH 7. In future, it should be possible to obtain tailor-made, but natural yellow fungal colorants by manipulating media and culture conditions.

**Table 1** and **Figure 3** illustrate a more qualitative aspect of decolorization; how the color hues of the orange-red pigment extracts and the control MR3 differed initially and after the light exposure as a function of time. The a* and the b* values of the control were relatively higher and lower, respectively, compared to all of the sample pigment extracts resulting in a hue angle of 34 indicating more red a hue than orange as in case of the sample pigment extracts (**Table 1, Figure 3**). The hue values of the pigment extract in both the food systems at pH 5 and 7 did not change much during the first two hours, then the path toward a weaker color was changed (**Figure 3**). The pigment extract in both the food systems at pH 5 did not show as much deviation as the pigment extract in the two food systems at pH 7 (**Figure 3**). Hence, at pH 7, a slight increase in the hue angles of the color in both of the two food systems at pH 7 was seen (**Table 1, Figure 3**), indicating a more yellow color hue. This could be due to the degradation of the red components

as explained earlier. This result is in agreement with the result obtained by Jung *et al*. (*10*) in case of the decoloration of control red *Monascus* pigments under sunlight irradiation.

**Chemistry of photodegradation of the orange-red pigment extract.** In order to see and understand the photodegradation pattern of the most photostable system i.e. the soft drink medium at pH 7, the pigment composition of the two food systems before and after the light exposure was analyzed by LC-DAD-MS. There were 2 minor orange-red colored components and 2 yellow components present with characteristic UV-vis spectra in the soft drink medium at pH 7 before the light exposure (**Figure 4, panel A**). Among the yellow components there was a major peak eluting at the retention time (RT) of 10.45 minutes and a minor peak with RT of 11.00 (**Figure 4, panel A**). After the light exposure, the peaks indicating the orange-red pigments disappeared, indicating that these minor orange-red components degraded to other compounds. In case of yellow components, the height of the major peak with RT 10.44 also decreased after light exposure while the height of the minor peak with RT of 10.99 increased (**Figure 4, panel B**). The yellow major peak with RT 10.45 was identified to be sequoiamonascin C (**Figure 4 panel A** and **C**) on the basis of the mass and the UV-vis spectra (**Figure 4, A1/B1** and **C1**). Sequoiamonascin C belongs to a series of fungal metabolites, sequoiamonascins A-D, which were previously reported to be produced by *Aspergillus parasiticus* (*16*). The UV-vis and mass spectral data of sequoiamonascin C matched quite well with the reported data (*16*) and the data available in the database such as Antibase (Wiley-VCH, Weinheim, Germany). The structure of sequoiamonascin C (**Figure 1**) revealed its similarity to a related polyketide based *Monascus* pigment ankaflavin the difference being in the -COOH moiety instead of a methyl group attached to the six-membered ring and the presence of a hydroxyl group instead of a keto group in the aliphatic chain as highlighted in **Figure 1.** This structural similarity may explain the similarity of their UV-vis spectra. The minor peak, RT 11.00 present in **Figure 4, panel A** is envisaged to be a structural homologue of sequoiamonascin C as it had the same mass spectrum but slightly different UV-vis spectrum (**Figure 4, B2**). Sequoiamonascin C, without its structural analogue, was also seen in the crude

pigment extract alone (data not shown). Sequoiamonascin D is reported to be produced by an unidentified species of *Penicillium* (*17*). This is a first ever report of sequoiamonascin C to be produced by *Penicillium aculeatum* IBT 14263 on YES medium and brings out the discovery of yet another *Monascus*-like pigment in *Penicillium aculeatum* IBT 14263. The chemistry behind the presence of structural homologue of sequoiamonascin C in the soft drink medium at pH 7 and their inter conversion upon light exposure can be explained as follows: Sequoiamonascin C has a log D value (used as lipophilicity index) of -0.24 at pH 7 (SciFinder Scholar Compound registry # 561305-71-3) meaning that it is quite hydrophilic but at the same time its low pKa value (4.84) resulted in its ionization in the soft drink medium at pH 7 giving rise to its structural analogue that coexisted before the light exposure. The light exposure induced a modification in the structure possibly with regards to the arrangement of the conjugated double bonds as exhibited by the resulting slight change in the UV-vis spectrum (**Figure 4, B2**). The weaker color after the light exposure was due to the presence of only one major yellow colored component with the area underlying the visible spectrum being small. Also a few tiny peaks were observed after the light exposure (**Figure 4, panel B**). These peaks could be the degradation products of the red-orange components present before the light exposure. Similar results were obtained in case of citrate buffer at pH 7 confirming the pH effect.

**Quantitative and qualitative color change of yellow fungal pigment extract with turmeric as control under light in soft drink medium and citrate buffer at two different pH. Figure 5** shows that the magnitude of color change of the pigment extract obtained from *Epicoccum nigrum* IBT 41028 on PD in the two food systems was not as large as compared to the orange-red pigment extract with respect to the change in pH of the food systems. Also the two food systems did not differ much from each other with respect to the extent of photodecolorization. The striking feature was that the decoloration was not as pronounced as in the control extract of turmeric, which decolorized (**Figure 5**) significantly after 1 hour. Turmeric, the color of which mainly comes from the compound curcumin (**Figure 1**), is one of the weakest light-stable colorants currently being used and thus needs to be encapsulated (*18*).

**Table 2** and **Figure 6** depict the qualitative change in the color over the time in citrate buffer at two different pH. It is important to note that the initial a*, b*, and C* values of the control (turmeric) for both pH 3 and pH 7 were significantly higher than the *E. nigrum* extracts (**Table 2**) even though the L* values were adjusted to the relatively similar level. We ascribe this difference due to the possibly higher extinction coefficient and pH stability of curcumin, the major component of the turmeric extract, than orevactaene. However, the a*, b*, and C* values of turmeric dropped drastically after 1 hour of light exposure compared to the extracts of *E. nigrum* (**Table 2**) indicating a weaker photostability of turmeric extract. The color hues, on the other hand, did not change significantly (especially at 0 hour) and were found to be similar for the extracts of *E. nigrum* as well as the control (**Table 2, Figure 6**). The color of the *E. nigrum* pigment extract at pH 3 was much weaker than at pH 7 at 0 hour and thus faded after 4 hours (**Table 2**). This could be due to the effect of concentration as it was not possible to have an equimolar concentration of pigments in the two food systems considering the crude nature of the fungal pigment extract. Nevertheless, the data signify the potential of *Epicoccum* pigment extract as a photostable colorant.

It must be noted that the intensity of irradiation/h in the cabinet (172.8 J/cm$^2$) we used for our experiments was 30-40 times higher than the regular intensity of sunlight irradiation/h in Denmark (personal communication Paul Eriksen, Danish Meteorological Institute, Dec. 2008). Also the intensity of light used under storage conditions of food products could be even lower. This implies that the time for decoloration in the real scenario would be much longer than the current experimental findings. Jung *et al.* (*10*) reported the enhanced photostability of amino acid derivatives of *Monascus* pigments under both UV light (365 nm, 8 W) and outdoor sunlight irradiation with the average intensity of sunlight irradiation/h ranging from 230-313 J/cm$^2$, which is higher than our experimental value. They found the amino acid derivatives of *Monascus* pigments much more stable compared to their parent red pigment under UV light than under sunlight possibly due to the lower irradiation strength of the UV light than sunlight they used. Since wavelength distribution of sunlight irradiation was not mentioned by Jung *et al.* (*10*), it is

thus difficult to directly correlate our results with the results obtained by them. Nevertheless, it is very likely that *Monascus* pigments and their amino acid derivatives may be more photostable than some of the existing natural colorants. Thus, the relatively higher photostability of both of the fungal pigment extracts compared to the controls is worth considering. Notably, the color hue of turmeric is similar to the pigment extract of *Epicoccum nigrum* IBT 41028 on PD as previously described (*7*). The yellow *Epicoccum* pigment extract, thus, harbors potential additional yellow colorants with better photostability.

**Chemistry of photodegradation of the pigment extract obtained from *Epicoccum nigrum* IBT 41028 on PDA.** The color of the pigment extract of *Epicoccum nigrum* IBT 41028 on PD was predominantly due to the presence of an oxopolyene polyketide orevactaene (**Figure 1**) as reported previously (*19*). The effect of light on the degradation of *Epicoccum* pigment extract in citrate buffer at pH 3 and pH 7 was studied by chromatographically analyzing the pigment composition before and after the light exposure. Orevactaene is relatively more hydrophilic at pH 7 as its log D value at pH 7 is 1.17 (SciFinder Scholar Compound registry # 197631-20-2). Also being highly acidic (pKa 4.07 ± 0.19), it ionized more at pH 7 and was eluted more in the neutral/acidic than in the alkaline elute (indicated by its higher peak height) (**Figure 7, G** vs**. H**). But the compound is quite hydrophobic at pH 3 (log D value at pH 3 is 4.01, SciFinder Scholar Compound registry # 197631-20-2) and remained in unionized form as a result a relatively small peak is detected in both neutral/acidic and the alkaline elute. This might explain the lower chroma of the *Epicoccum* pigment extracts at pH 3. The retention time also changed from 12.07/12.08 to 12.27/12.29 which could be due to the change in the relative abundance of orevactaene isotopes. The effect of pH was also observed in the soft drink medium. After the light exposure, absence of orevactaene could be seen in both neutral/acidic elute and the alkaline elute extracted from citrate buffer at pH 3 and pH 7 (**Figure 7, E1-H1**).

In conclusion, we have shown two fungal pigment extracts exhibiting enhanced photostability compared to the controls in our study. The application based approach was shown to add a more realistic dimension in assessing the photostability using CIELAB color space. Yellow

components of the orange-red pigment extract from *Penicillium aculeatum* IBT 14263 were found to be more light-stable than the red components. In addition, yet another *Monascus*-like pigment sequoiamonascin C was discovered in the extract of *Penicillium aculeatum* IBT 14263 on YES medium. The data indicate that ascomycetous fungi may provide a significant source of new, natural food colorants with desirable functionalities that may even surpass those of currently used natural colorants.

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Table 1 Colorimetric values for the decoloration of the orange-red fungal extracts from *Penicillium aculeatum* IBT 14263 and control *Monascus* Red (MR3) colorant[1] in the soft drink medium (SD) and 0.1 M citrate buffer (CB) as a function of time under the light exposure[2]

| Color coordinates | Sample name | Time (in hours) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **0** | **1** | **2** | **4** | **5** | **6** | **8** | **9** |
| | 14263 CB pH 5 | 75.24 | 77.72 | 80.62 | 85.90 | Nd[3] | 88.32 | 90.91 | 91.62 |
| | 14263 CB pH 7 | 75.44 | 76.12 | 78.07 | 81.97 | Nd[3] | 83.13 | 84.96 | 85.14 |
| **L*** | 14263 SD pH 5 | 75.64 | 78.75 | 81.5 | 86.15 | Nd[3] | 88.17 | 91.01 | 91.86 |
| | 14263 SD pH 7 | 75.10 | 75.53 | 77.15 | 80.44 | Nd[3] | 80.72 | 82.05 | 82.37 |
| | MR3 SD pH 7 | 76.07 | 80.59 | Nd[3] | Nd[3] | 91.51 | Nd[3] | Nd[3] | 96.18 |
| | MR3 CB pH 7 | 76.78 | 81.34 | Nd[3] | Nd[3] | Nd[3] | Nd[3] | Nd[3] | 96.21 |
| | 14263 CB pH 5 | 31.01 | 24.13 | 19.82 | 13.44 | Nd[3] | 10.63 | 7.38 | 6.36 |
| | 14263 CB pH 7 | 31.18 | 27.19 | 23.81 | 17.42 | Nd[3] | 14.72 | 12.02 | 11.67 |
| **a*** | 14263 SD pH 5 | 30.37 | 23.45 | 19.63 | 14.15 | Nd[3] | 11.82 | 8.5 | 7.54 |
| | 14263 SD pH 7 | 31.73 | 28.57 | 25.49 | 19.25 | Nd[3] | 18.33 | 16.85 | 16.47 |
| | MR3 SD pH 7 | 38.74 | 30.77 | Nd[3] | Nd[3] | 16.32 | Nd[3] | Nd[3] | 3.41 |
| | MR3 CB pH 7 | 36.34 | 29.38 | Nd[3] | Nd[3] | Nd[3] | Nd[3] | Nd[3] | 3.38 |
| | 14263 CB pH 5 | 39.38 | 29.75 | 26.27 | 20.31 | Nd[3] | 15.67 | 13.25 | 12.16 |
| | 14263 CB pH 7 | 42.59 | 32.59 | 30.45 | 29.61 | Nd[3] | 27.03 | 25.54 | 24.98 |
| **b*** | 14263 SD pH 5 | 37.55 | 27.39 | 24.14 | 19.17 | Nd[3] | 15.12 | 12.46 | 11.54 |
| | 14263 SD pH 7 | 41.66 | 32.96 | 30.40 | 29.79 | Nd[3] | 27.53 | 26.70 | 26.23 |
| | MR3 SD pH 7 | 26.01 | 23.92 | Nd[3] | Nd[3] | 17.23 | Nd[3] | Nd[3] | 8.14 |
| | MR3 CB pH 7 | 26.55 | 24.74 | Nd[3] | Nd[3] | Nd[3] | Nd[3] | Nd[3] | 8.18 |
| | 14263 CB pH 5 | 48.29 | 38.31 | 32.91 | 24.35 | Nd[3] | 18.94 | 15.17 | 13.72 |
| | 14263 CB pH 7 | 52.78 | 42.44 | 38.65 | 34.35 | Nd[3] | 30.78 | 28.23 | 27.57 |
| **C*** | 14263 SD pH 5 | 48.29 | 36.06 | 31.11 | 23.83 | Nd[3] | 19.19 | 15.08 | 13.78 |
| | 14263 SD pH 7 | 52.37 | 43.62 | 39.67 | 35.47 | Nd[3] | 33.07 | 31.57 | 30.97 |
| | MR3 SD pH 7 | 46.66 | 38.97 | Nd[3] | Nd[3] | 23.73 | Nd[3] | Nd[3] | 8.83 |
| | MR3 CB pH 7 | 45.01 | 38.41 | Nd[3] | Nd[3] | Nd[3] | Nd[3] | Nd[3] | 8.85 |
| | 14263 CB pH 5 | 51.03 | 50.95 | 52.97 | 56.51 | Nd[3] | 55.85 | 60.88 | 62.39 |
| | 14263 CB pH 7 | 53.79 | 50.16 | 51.98 | 59.53 | Nd[3] | 61.43 | 64.8 | 64.96 |
| **H*** | 14263 SD pH 5 | 51.03 | 49.43 | 50.88 | 53.57 | Nd[3] | 51.98 | 55.70 | 56.84 |
| | 14263 SD pH 7 | 52.71 | 49.08 | 50.02 | 57.13 | Nd[3] | 56.34 | 57.74 | 57.88 |
| | MR3 SD pH 7 | 33.88 | 37.86 | Nd[3] | Nd[3] | 46.55 | Nd[3] | Nd[3] | 67.27 |
| | MR3 CB pH 7 | 36.15 | 40.1 | Nd[3] | Nd[3] | Nd[3] | Nd[3] | Nd[3] | 67.55 |

[1] The data for control Monascus Red (MR3) colorant for both CB and SD at pH 5 could not be recorded as it showed signs of degradation at 0 hour itself, [2] Xenon lamp, light intensity/h 172.8 J/cm$^2$, cut-on wavelength 250 nm, [3] Not determined

Table 2. Colorimetric values for the decoloration of the yellow fungal extract from *Epicoccum nigrum* IBT 41028 and control (Turmeric) in 0.1 M citrate buffer (CB) as a function of time under the light exposure[1]

| Color coordinates | Sample name | Time (in hours) | | | | | |
|---|---|---|---|---|---|---|---|
| | | **0** | **1** | **2** | **4** | **6** | **10** |
| | Turmeric CB pH 3 | 97.94 | 99.55 | 99.61 | 99.68 | - | - |
| | *E. nigrum* CB pH 3 | 98.17 | 99.42 | 99.46 | 99.54 | - | - |
| **L*** | Turmeric CB pH 7 | 97.73 | 98.63 | 98.67 | 98.59 | 98.60 | 98.40 |
| | *E. nigrum* CB pH 7 | 97.45 | 98.09 | 97.80 | 98.57 | 98.77 | 99.23 |
| | Turmeric CB pH 3 | -20.72 | -3.73 | -1.94 | -1.16 | - | - |
| | *E. nigrum* CB pH 3 | -3.26 | -0.58 | -0.45 | -0.35 | - | - |
| **a*** | Turmeric CB pH 7 | -19.86 | -4.11 | -2.33 | -1.49 | -1.01 | -0.48 |
| | *E. nigrum* CB pH 7 | -11.33 | -10.67 | -10.00 | -9.01 | -7.75 | -4.03 |
| | Turmeric CB pH 3 | 91.17 | 10.28 | 6.01 | 3.98 | - | - |
| | *E. nigrum* CB pH 3 | 16.54 | 3.24 | 2.64 | 2.07 | - | - |
| **b*** | Turmeric CB pH 7 | 86.87 | 15.00 | 10.02 | 7.40 | 6.12 | 4.84 |
| | *E. nigrum* CB pH 7 | 43.79 | 36.56 | 33.30 | 28.56 | 23.85 | 12.30 |
| | Turmeric CB pH 3 | 93.49 | 10.94 | 6.32 | 4.15 | - | - |
| | *E. nigrum* CB pH 3 | 16.86 | 3.29 | 2.68 | 2.10 | - | - |
| **C*** | Turmeric CB pH 7 | 89.11 | 15.55 | 10.29 | 7.55 | 6.20 | 4.86 |
| | *E. nigrum* CB pH 7 | 45.23 | 38.09 | 34.77 | 29.95 | 25.08 | 12.94 |
| | Turmeric CB pH 3 | 102.8 | 109.94 | 107.89 | 106.25 | - | - |
| | *E. nigrum* CB pH 3 | 101.15 | 100.15 | 99.67 | 99.60 | - | - |
| **H*** | Turmeric CB pH 7 | 102.88 | 105.32 | 103.09 | 101.38 | 99.37 | 95.66 |
| | *E. nigrum* CB pH 7 | 104.51 | 106.27 | 106.72 | 107.51 | 108.00 | 108.14 |

[1] Xenon lamp, light intensity/h 172.8 J/cm$^2$, cut-on wavelength 250 nm, "-" indicates the color was too faint to be measured.

FIGURE Legends/Captions

**Figure 1**. Structures of pigments relevant for the present work (Formula and nominal masses in parentheses, highlighted area in 1 and 2 show their structural difference, 2 detected and identified in the present work)

**Figure 2**. Quantitative decoloration over time of *Penicillium aculeatum* IBT 14263 pigment extract (orange-red) in soft drink medium and 0.1 M citrate buffer at pH 5 and 7 with commercially available *Monascus* Red (MR3) colorant as control under light exposure (Xenon lamp, light intensity/h 172.8 J/cm$^2$, cut-on wavelength 250 nm). Soft drink medium is designated as SD and citrate buffer is designated as CB. The data for control for both CB and SD at pH 5 could not be recorded as it showed signs of degradation at 0 hour itself. $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$, where $\Delta L^*$ is change in the lightness value with respect to time, $\Delta a^*$ is change in the red-green CIELAB coordinate a* with respect to time, and $\Delta b^*$ is change in the yellow-blue CIELAB coordinate b* with respect to time.

**Figure 3**. Qualitative color change over time in CIELAB color space for *Penicillium aculeatum* IBT 14263 pigment extract (orange-red) in soft drink medium and 0.1 M citrate buffer at pH 5 and 7 with commercially available *Monascus* Red (MR3) colorant as control under light exposure (Xenon lamp, light intensity/h 172.8 J/cm$^2$, cut-on wavelength 250 nm). Soft drink medium is designated as SD and citrate buffer is designated as CB. The data for control for both CB and SD at pH 5 could not be recorded as it showed signs of degradation at 0 hour itself.

**Figure 4**. Bottom panel (A-B) depicts UV-vis chromatograms of 390-700 nm of neutral/acidic fraction of pigment extract obtained from *Penicillium aculeatum* IBT 14263 on YES medium in soft drink medium at pH 7. A. UV-vis chromatogram before the light exposure. B. UV-vis chromatogram after the light exposure. A1/B1 shows the UV-vis spectrum of Sequoiamonascin C. A2 shows the UV-vis spectrum of one of the orange-red components. B2 represents UV-vis spectrum of the proposed structural analogue of Sequoiamonascin C. Top panel (C-D) depicts total ion chromatograms (m/z 100-900) from positive ion electrospray of the same fraction. C.

Total ion chromatogram (m/z 100-900) before the light exposure. D. Total ion chromatogram (m/z 100-900) after the light exposure. C1 depicts the mass spectrum of sequoiamonascin C. D1 represents mass spectrum of its structural analogue.

**Figure 5**. Quantitative decoloration over time of *Epicoccum nigrum* IBT 41028 pigment extract (yellow) in soft drink medium and 0.1 M citrate buffer at pH 5 and 7 with commercially available turmeric colorant as control under light exposure (Xenon lamp, light intensity/h 172.8 J/cm$^2$, cut-on wavelength 250 nm). Soft drink medium is designated as SD and citrate buffer is designated as CB. $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$, where $\Delta L^*$ is change in the lightness value with respect to time, $\Delta a^*$ is change in the red-green CIELAB coordinate a* with respect to time, and $\Delta b^*$ is change in the yellow-blue CIELAB coordinate b* with respect to time.

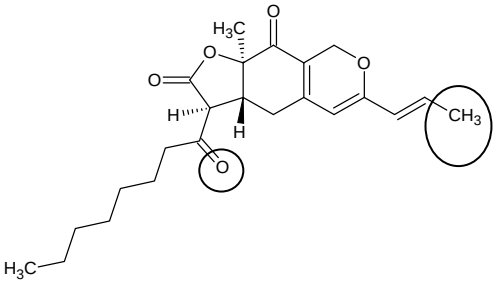
**Figure 6**. Qualitative color change in CIELAB color space of *Epicoccum nigrum* IBT 41028 pigment extract (yellow) in 0.1 M citrate buffer at pH 3 and 7 with commercially available turmeric colorant as control under light exposure (Xenon lamp, light intensity/h 172.8 J/cm$^2$, cut-on wavelength 250 nm). Citrate buffer is designated as CB.

**Figure 7**. UV-vis chromatograms of 390-700 nm of pigment extract obtained from *Epicoccum nigrum* IBT 41028 on PD medium in 0.1 M citrate buffer at pH 3 and 7. Data normalized to the intensity of $1.0 \times 10^6$ to focus on the major peak orevactaene. E. Presence of orevactaene in citrate buffer at pH 3 neutral/acidic elute before the light exposure, E1 Absence of orevactaene after the light exposure; F. Presence of orevactaene in citrate buffer at pH 3 alkaline elute before the light exposure, F1 Absence of orevactaene after the light exposure; G Citrate buffer at pH 7 neutral/acidic elute before light exposure, G1 Absence of orevactaene after the light exposure; H. Presence of orevactaene in citrate buffer at pH 7 alkaline elute before the light exposure, H1 Absence of orevactaene after the light exposure.

**Figure1**.

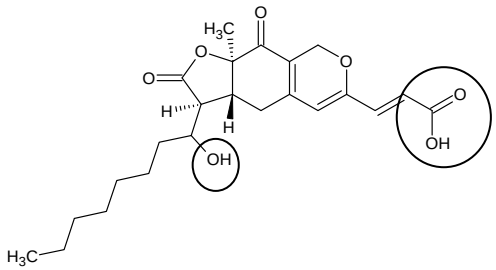
**A. <u>Pigments</u>**

**1. Ankaflavin**



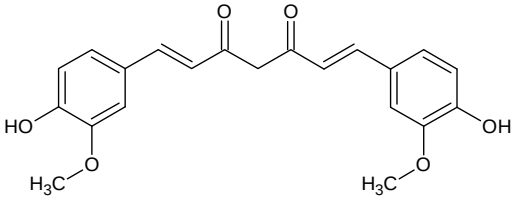
*($C_{23}H_{30}O_5$, 386.48)*

**2. Sequoiamonascin C**



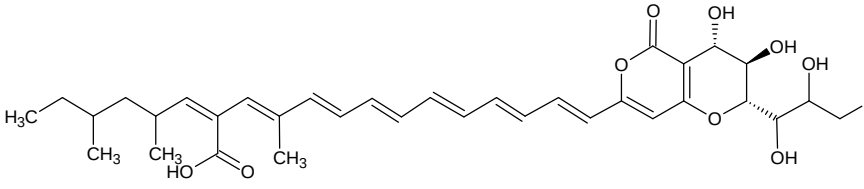
*($C_{23}H_{30}O_7$, 418.48)*

**3. Curcumin**



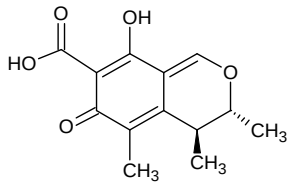
*($C_{21}H_{20}O_6$, 368.38)*

**4. Orevactaene**



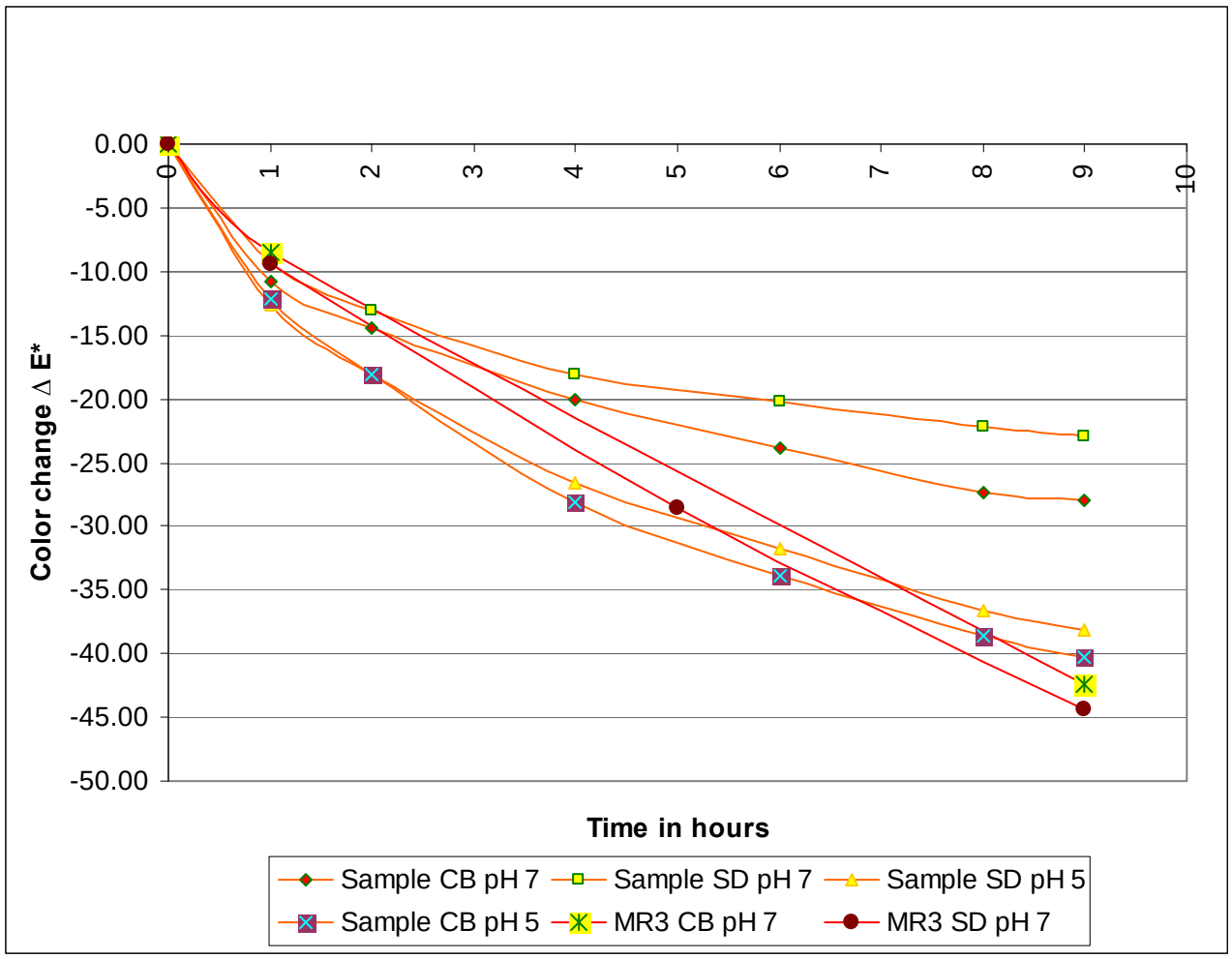
*($C_{34}H_{44}O_{10}$, 612.70)*

**B. <u>Toxic metabolite</u> Citrinin**



*($C_{13}H_{14}O_5$, 250.25)*

**Figure 2**


0.00
-5.00
-10.00
-15.00
-20.00
-25.00
-30.00
-35.00
-40.00
-45.00
-50.00
0
1
2
3
4
5
6
7
8
9
10
Color change Δ E*
Time in hours
Sample CB pH 7
Sample SD pH 7
Sample SD pH 5
Sample CB pH 5
MR3 CB pH 7
MR3 SD pH 7

## Figure 3

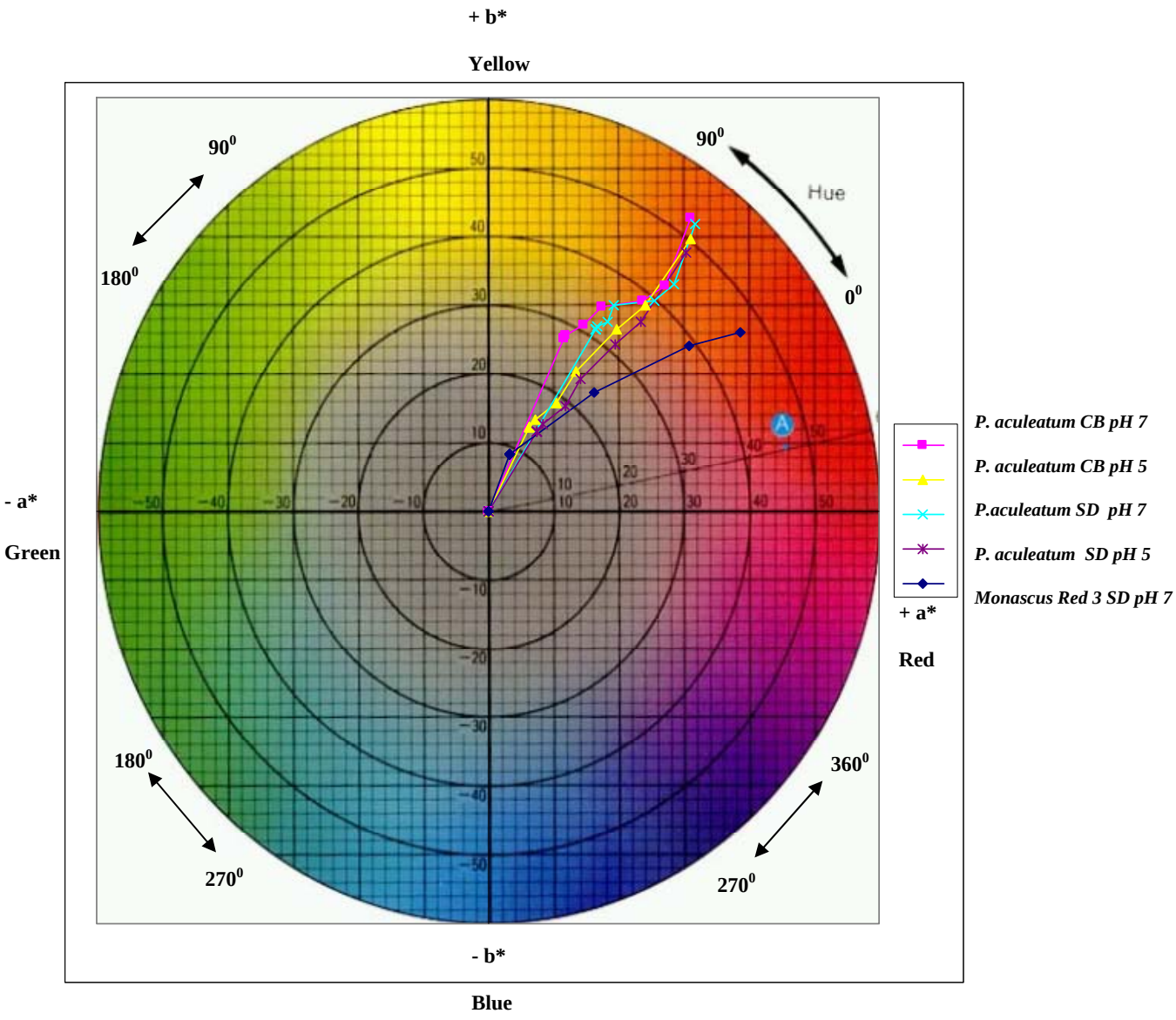

+ b*
Yellow
90⁰
90⁰
Hue
180⁰
0⁰
- a*
Green
+ a*
Red
180⁰
360⁰
270⁰
270⁰
- b*
Blue
P. aculeatum CB pH 7
P. aculeatum CB pH 5
P.aculeatum SD pH 7
P. aculeatum SD pH 5
Monascus Red 3 SD pH 7

**Figure 4**

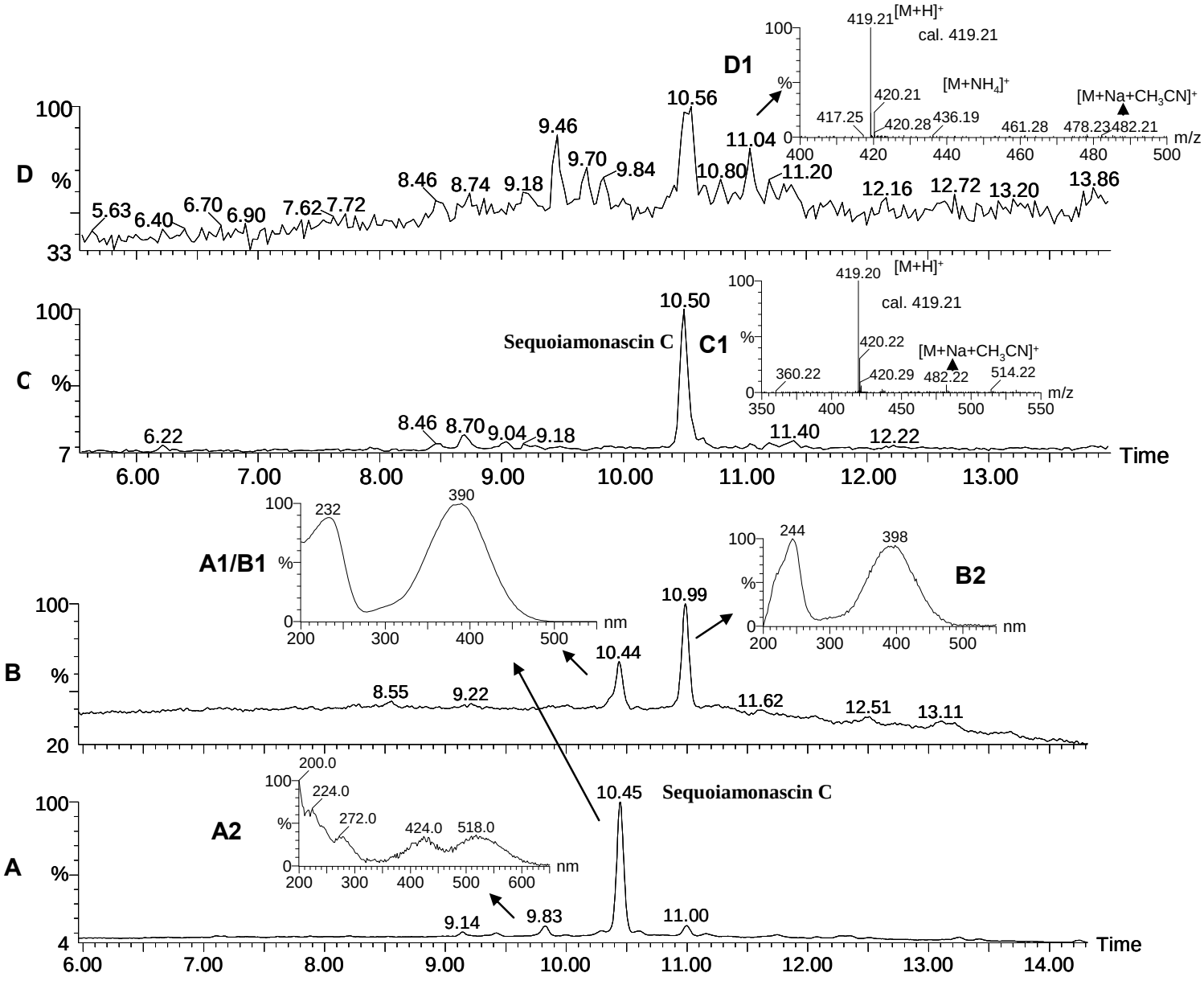
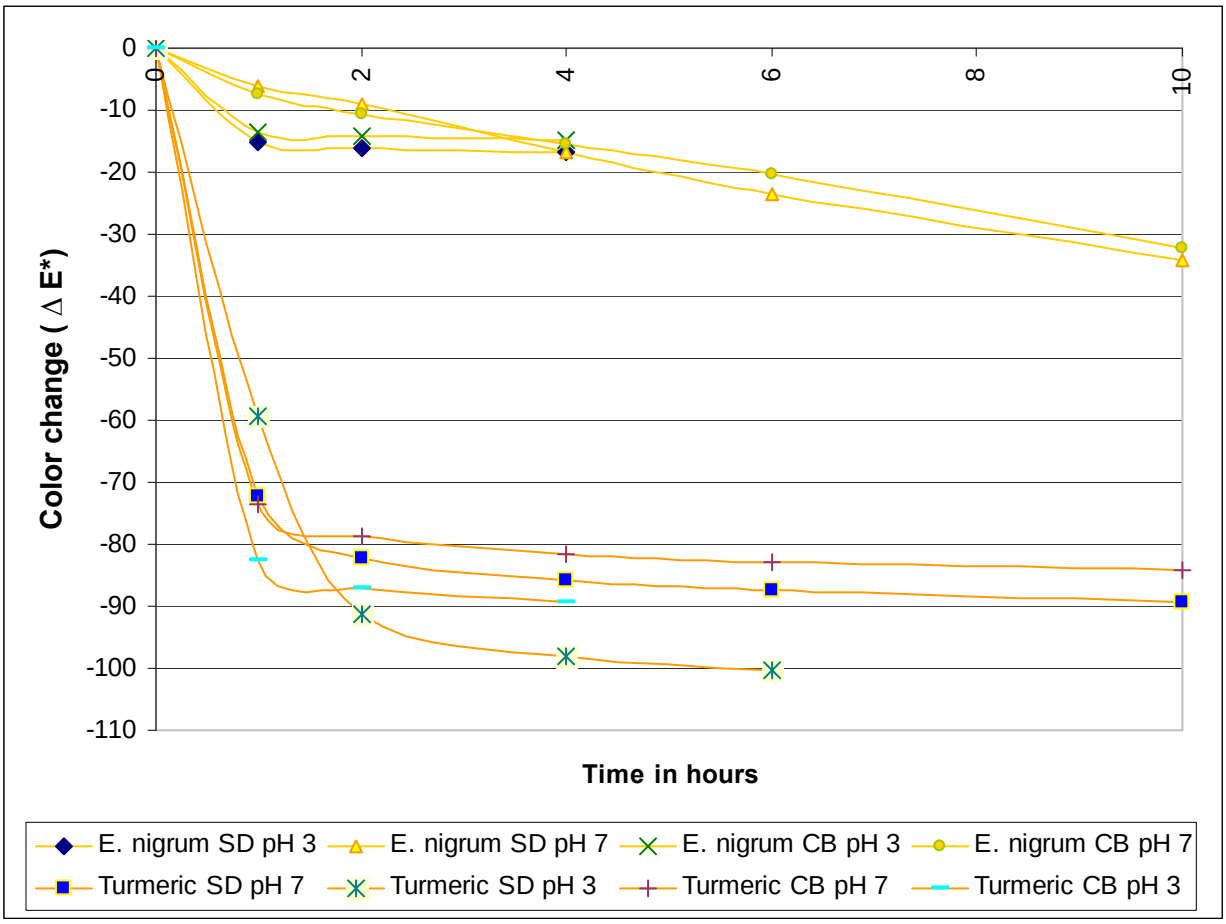

D
D1
419.21 [M+H]+
cal. 419.21
[M+NH4]+
[M+Na+CH3CN]+
10.56
9.46
9.70
9.84
11.04
10.80
11.20
8.46
8.74
9.18
12.16
12.72
13.20
13.86
5.63
6.40
6.70
6.90
7.62
7.72
C
C1
Sequoiamonascin C
10.50
419.20 [M+H]+
cal. 419.21
[M+Na+CH3CN]+
6.22
8.46
8.70
9.04
9.18
11.40
12.22
Time
A1/B1
232
390
B
B2
244
398
10.99
10.44
8.55
9.22
11.62
12.51
13.11
A
A2
200.0
224.0
272.0
424.0
518.0
10.45
Sequoiamonascin C
9.14
9.83
11.00
Time
nm

**Figure 5.**


0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
0
2
4
6
8
10
Color change ( Δ E*)
Time in hours
E. nigrum SD pH 3
E. nigrum SD pH 7
E. nigrum CB pH 3
E. nigrum CB pH 7
Turmeric SD pH 7
Turmeric SD pH 3
Turmeric CB pH 7
Turmeric CB pH 3

## Figure 6

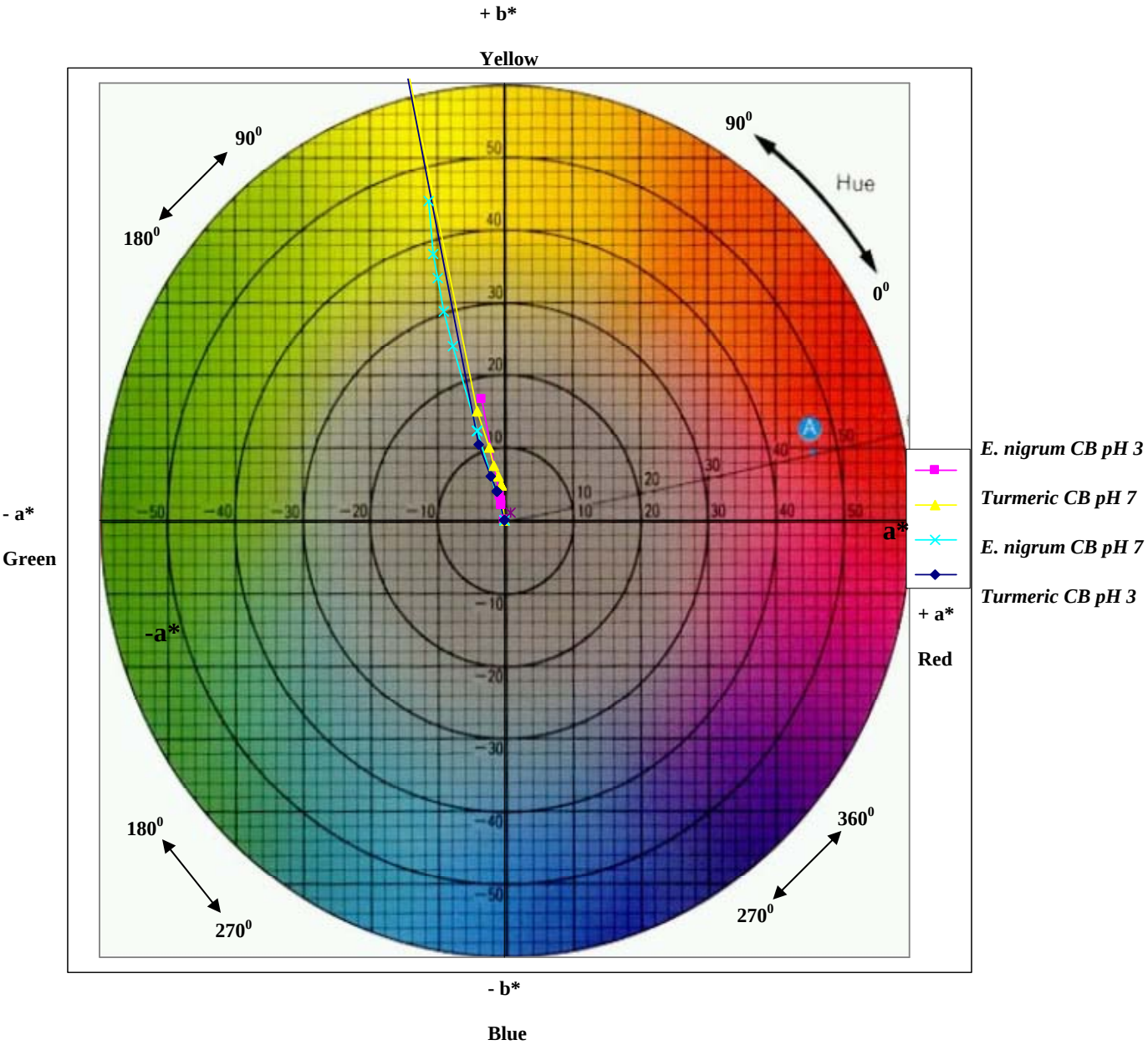
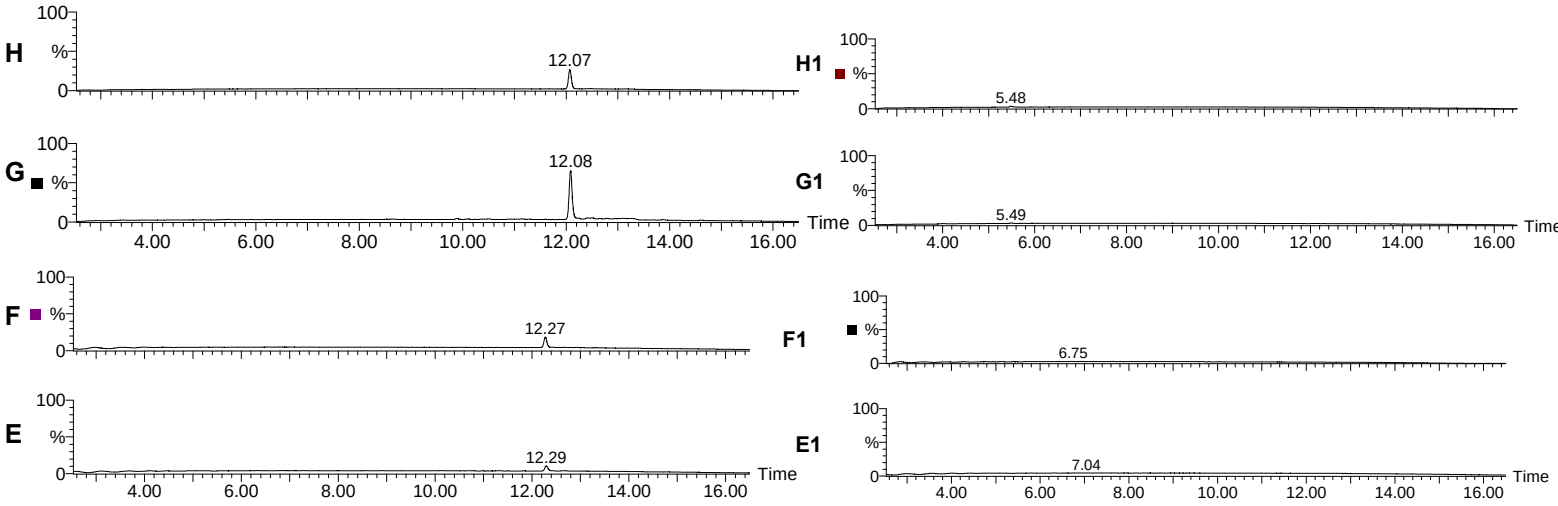

+ b*
Yellow
90⁰
180⁰
90⁰
Hue
0⁰
- a*
Green
a*
-a*
+ a*
Red
180⁰
270⁰
360⁰
270⁰
- b*
Blue
E. nigrum CB pH 3
Turmeric CB pH 7
E. nigrum CB pH 7
Turmeric CB pH 3

**Figure 7**.


H
12.07
H1
5.48
G
12.08
G1
5.49
F
12.27
F1
6.75
E
12.29
E1
7.04
100
%
0
4.00
6.00
8.00
10.00
12.00
14.00
16.00
Time

# *Chapter 7*

## *Evaluation of Potentially Safe Promising Fungal Cell Factories for the Production of Polyketide Natural Food Colorants*

# Chapter 7: Evaluation of potentially safe promising fungal cell factories for the production of polyketide natural food colorants

This chapter is based on two manuscripts. First part is based on the manuscript entitled "**Evaluation of Epicoccum nigrum for growth, morphology and production of natural colorants in liquid media and on a solid rice medium"**.

Sameer AS Mapari, Anne S. Meyer, and Ulf Thrane.

*Biotechnology Letters* (**2008**) **30**: 2183-2190, Copyright [2008] Springer

The second part is partly based on the manuscript entitled "**Identification of potentially safe promising fungal cell factories for the production of polyketide natural food colorants using chemotaxonomic rationale**"

Sameer AS Mapari, Anne S. Meyer, Ulf Thrane, and Jens C. Frisvad.

*Microbial Cell Factories* 2009, **8**:24

The published form of the full manuscript can be found in Appendix A. For a good flow of the thesis, full manuscript is not presented here.

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DOI 10.1007/s10529-008-9798-y

ORIGINAL RESEARCH PAPER

# Evaluation of *Epicoccum nigrum* for growth, morphology and production of natural colorants in liquid media and on a solid rice medium

**Sameer A. S. Mapari · Anne S. Meyer · Ulf Thrane**

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**Abstract** Four nonpathogenic and nontoxigenic *Epicoccum nigrum* strains were evaluated for their growth, morphology and pigment producing ability in three complex and one defined liquid media. *Epicoccum nigrum* IBT 41028 produced pigments in all the four media tested with a maximum pigment of 3.68 AU at 410 nm in M1 medium (unoptimized) containing 5 g/l yeast autolysate. The color hue of the crude pigment extracts ranged from 74 to 102 exhibiting dark orange to green-yellow color. Pelleted morphology was shown to have a positive influence on the pigment production by *E. nigrum* strain IBT 41028 in the liquid media, and the use of Bis-tris buffer was found to diminish or reduce the pellet formation. Since *Monascus* is a well known pigment producer on rice. Pigment producing ability of *E. nigrum* IBT 41028 was tested on rice and compared to liquid media with *Monascus ruber* IBT 7904 as control. Though, both genera preferred rice but *E. nigrum* produced 4.6 folds higher pigment in the liquid unoptimized fermentation medium compared to *M. ruber*. Solid phase extraction and subsequently HPLC-DAD analysis of the crude pigment extracts showed qualitative as well as quantitative variation in the pigment composition under solid and liquid cultivations.

**Keywords** *Epicoccum nigrum* · *Monascus* · Morphology · Natural colorant · Polyketide pigments · Rice medium

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## Introduction

There is a growing preference for natural colorants among consumers because of their manifold advantages over synthetic colorants in terms of both health and the environment (Kumar and Sinha 2004). Among microbial sources of natural colorants, micro-algae and filamentous fungi are the most explored ones with the former have a limitation of low productivity. Filamentous fungi, on the other hand, present a readily available potential alternative or additional source to the existing sources of natural colorants owing to their amenability to be produced in higher yields with the available cultivation technology. Such a production system would be independent of the availability and external supply of raw materials unlike in case of the production of plants or insects based natural colorants that are currently in use (Mapari et al. 2005). Fungal pigments can be classified as carotenoids and/or structurally diverse polyketide class of pigments

produced as secondary metabolites, the example of latter kind are *Monascus* pigments that have been known for hundreds of years as natural food colorants (Dufosse et al. 2005). Previous studies have brought out the potential of pigment-producing genera other than *Monascus*, the colors of which resemble some of the commercially available food colorants especially in the yellow spectrum (Mapari et al. 2006). *Epicoccum* is one such a genus which has been previously reported to produce a variety of secondary metabolites including pigments of both carotenoids (Foppen and Gribanovski-Sassu 1968; Gribanovski-Sassu et al. 1970; Burge et al. 1976) and/or polyketide origin (Shu et al. 1997) with color hues in red-orange-yellow spectra; water soluble yellow pigment with antioxidant property was reported by Stricker et al. (1981).

Secondary metabolite producing ability of filamentous fungi is facilitated on solid substrates as they are the natural habitat for most fungal species. This has triggered the interest in solid substrate fermentation (SSF) for a range of fungal secondary metabolites (Barrios-Gonzalez and Mejia 1996; Pandey et al. 1999; Medeiros et al. 2001). On the other hand, industry favors the use of submerged liquid fermentation (SLF) owing to its easier product recovery and purification, and the use of standardized conditions in terms of culture media choice and typical fermentation parameters (Aldfred et al. 2005). Despite the choice of fermentation, it is important to understand the functioning of the entire production system including the micro and the macro environment around the production organism, its morphology and the choice of substrate that can be manipulated to get the maximum of the desired product. The relationship between fungal morphology and metabolite production in filamentous fungi is well documented (Clark and Lentz 1963; Su and He 1997; Paul and Thomas 1996). The production of some fungal metabolites is facilitated by dispersed hyphae as in case of glucoamylase production by *Aspergillus awamori* (Ruohang and Webb 1995) and some, for instance, high $\beta$-glucan production by *Acremonium persicinum*, are associated with a pelleted morphology (Stasinopoulos and Seviour 1992). Therefore, it is important to consider the morphology that favors the formation of a desired product in order to envisage the design of the fermentation equipment for an efficient and economic production system. For the pigment production by *E. nigrum*, there are no reports available that give an insight on the response of the fungus towards growth and production in the liquid media and on rice. This would come handy as a pre requisite for an eventual large scale set up for the production of natural colorants derived by the fungus *E. nigrum.*

In the light of this, we have studied the pigment production by four strains of *E. nigrum*, preselected based on chemotaxonomic knowledge, in the previously reported three complex and one chemically defined liquid media. The objective of this study was to evaluate the qualitative growth and pigment production of *E. nigrum* strains and also to investigate if a particular morphology favors the pigment production. In addition, we also examined if *E. nigrum* can grow and produce pigments on rice with *Monascus ruber* as a control and have compared to its pigment producing ability in the liquid media. The pigment compositions of crude color extracts obtained by both SLF and SSF were analyzed by high-resolution liquid chromatography-diode array detection (LC-DAD) to see the variation in pigment profile, if any, obtained by the two cultivation methods.

## Materials and methods

### Strains

Four strains of *E. nigrum* viz. IBT 7571, IBT 7802, IBT 7901, and IBT 41028 (pre-selected from our chemotaxonomic solid screening) and one strain of *M. ruber* (IBT 7904) was used in this study. The strains were procured from the IBT Fungal Culture Collection at the Department of Systems Biology, Technical University of Denmark, (Kgs. Lyngby, Denmark), and listed by their respective IBT numbers.

### Media and culture conditions

#### *Initial liquid screening*

Medium 1 [M1] was composed of 1% (w/v) glucose, and 0.5% (w/v) yeast extract.

Medium 2 (modified Czapeck-Dox) [M2] consisted of 3% (w/v) glucose, 0.3% (w/v) $NaNO_3$, 0.1% of $KH_2PO_4$, and 1% trace metal solution (Samson et al. 2002).

Medium 3 [M3] comprised of 2% (w/v) glucose, 2% (w/v) malt extract, and 0.1% (w/v) peptone. Medium 4 [M4] was dehydrated potato/dextrose broth and reconstituted as per the manufacturer's instructions.

Two hundred mM of Bis-tris buffer (Sigma) was added to M1 and M3 medium wherever mentioned, to give 50 mM.

Five hundred ml baffled Erlenmeyer flasks with a working volume of 100 ml were used for the liquid screening. The shake-flasks were inoculated with 10 mycelial plugs of approx 6 mm diameter from a week-old culture grown on potato/dextrose/agar (Samson et al. 2002) plate. Incubation was carried out at 25°C in the dark on a rotary shaker at 150 rpm for 7 days.

*Solid and submerged cultivations*

Inoculum media was composed of glucose 5% (w/v), peptone 2% (w/v), $KH_2PO_4$ 0.8% (w/v), potassium acetate 0.05% (w/v), NaCl 0.2% (w/v), and $MgSO_4 \cdot 7H_2O$ 0.05% (w/v).

Fermentation solid media comprised of 20 g long grain rice (purchased locally in a supermarket) wetted with 20 ml 0.015 M $ZnSO_4$.

Fermentation liquid media was composed of glucose 5% (w/v), $NH_4Cl$ 1% (w/v), $KH_2PO_4$ 0.1% (w/v), $MgSO_4 \cdot 7H_2O$ 0.05% (w/v), KCl 0.05% (w/v), and $FeSO_4$ 0.001% (w/v). The initial pH was adjusted to 6.6 with 0.1 N NaOH prior to sterilization.

For inoculum build up, mycelia were scraped off from 5 days-old actively growing cultures and a 2% (w/v) of pre-seed in sterile distilled water was used as an inoculum for the seed medium. After 48 h of incubation under the previously mentioned conditions, a 5% (v/v) of inoculum was added into the main fermentation media.

Extraction methods

Total pigments were extracted as per the method of Jung et al. (2003) for both solid and liquid cultivations. On an average, three extractions were carried out, to extract maximum pigments. Solid phase extraction of the crude color extracts was carried out using a Strata-X-C 33 µm cation mixed mode polymer column (Phenomenex ApS, Allerød, Denmark) using 60 mg/3 ml. One ml of methanol was used for conditioning followed by 1 ml of distilled water for calibration. Samples, acidified with 0.1% phosphoric acid, were loaded and washed with 0.1% phosphoric acid. Neutral and acidic components were eluted with methanol and alkaline components were eluted with 5% (v/v) ammonia in methanol.

Analytical methods

*Colorimetry*

Total pigments were estimated from their absorption maxima. The same extracts were then used for determining CIELAB color coordinates using Chromameter (Minolta CT 310, Konica Minolta, Mahwah, USA). The CIELAB colorimetric system was interpreted as follows: L* indicates lightness read from 0 (black) to 100 (white). A positive a* value indicates red while a negative a* value represents green. Similarly positive and negative b* values indicate yellow and blue, respectively. Chroma values denote the saturation or purity of color. Hue angle values represent the degree of redness, yellowness, greenness and blueness the maximum being at 0, 90, 180, and 270, respectively.

*Chromatography*

Chromatographic analyses were performed using a 50 × 2 mm, i.d., 3 µm, Luna C18 II column (Phenomenex, Torrance, CA). The LC system was operated using a water/acetonitrile gradient system starting from 15% (v/v) acetonitrile, which was increased linearly to 100% in 20 min with a holding time of 5 min. Data analysis was done using Chemstation version B.01.01.

## Results and discussion

Pigment producing ability, the color range, and the morphology of *E. nigrum* strains in liquid media under shaking conditions

Four *E. nigrum* strains were used. The pre-selection was based on solid screening whereby they were found to produce relatively more pigment than the rest of the strains (data unpublished). Table 1 shows

**Table 1** Growth and pigment producing ability of *Epicoccum nigrum* in liquid media

| Strain | Media/[a]Type | Qualitative growth[b]/Morphology[c] | Pigment[d] |
|---|---|---|---|
| IBT 7571 | M1 | +++/Smooth pellets | – |
| | M2 | +/Hairy pellets | 0.4 ± 0.07 |
| | M3 | +++/Medium pellets | |
| | M4 | ++/Big floccules | – |
| IBT 7802 | M1 | ++/Big floccules | 2.78 ± 0.08 |
| | M2 | +/Spiky pellets | – |
| | M3 | ++/Mixed pellets | 2.47 ± 0.09 |
| | M4 | ++/Smooth pellets | 3.38 ± 0.12 |
| IBT 7901 | M1 | +/Smooth pellets | – |
| | M2 | +/Cottony pellets | – |
| | M3 | +/Big spiky pellets | – |
| | M4 | +/Small pellets | – |
| IBT 41028 | M1 | +++/Mixed pellets | **3.68 ± 0.08** |
| | M2 | +/Spiky pellets | 0.82 ± 0.10 |
| | M3 | ++/Small pellets | 2.95 ± 0.12 |
| | M4 | +/Mixed pellets | 1.03 ± 0.11 |

[a] M1, M3, and M4 were complex media, while M2 was defined medium

[b] The biomass was qualitatively assessed and rated on a scale of + to +++++ for the scanty growth to intense growth with an intermediate level represented by +++. Experiment was performed in duplicate

[c] Size of pellets were scaled from small: approximately 0.5 cm diameter, medium: approximately 1–2 cm diameter, and large: approximately >2 cm diameter

[d] Pigment producing ability was expressed in terms of absorbance at 410 nm. Average values of two determinations are shown with standard deviation. '–' indicates absence of pigment production. Bold indicates maximum pigment production

the results of the chosen strains after they were grown in the three complex viz. M1, M3, and M4 and one defined medium (M2) for 7 days; media were selected based on the scarcely available literature for the pigment production by *E. nigrum* in the liquid cultivations. All of the tested strains grew in all of the tested media. The biomass was qualitatively assessed and rated on a scale of + to +++++ for the scanty growth to intense growth with an intermediate level of +++. The striking feature observed was that the presence and/or the amount of biomass did not necessarily result in the pigment production as Table 1 shows that IBT 7571 did not produce pigments in M1 and M3 media even though there was considerable biomass in this media. Thus the growth and pigment-producing ability may not be positively correlated to each other in the liquid media for *E. nigrum*. Also, the pigment producing ability varied according to the media and the strains under study. For instance, IBT 41028 produced pigments in all the four media tested, IBT 7802 could produce pigment in all the complex media, IBT 7571 in the defined medium only, while IBT 7901 did not produce any pigment in all the four media.

Complex or defined media did not influence the pigment production for the strains under study; though the strain producing pigment in both kinds of media (Strain IBT 41028) produced a maximum (3.68 AU at 410 nm) in the complex M1 medium (Table 1). This indicates that pigment-producing ability is conferred by both genetic and the environmental factors; media being the crucial one. Though all the strains tested could produce pigments in the same solid media (data not shown), only IBT 41028 exhibited a relatively broader pigment-producing ability in the liquid media indicating a good substrate utilization range. The findings that the media affected both growth and pigment producing ability signified the importance of evaluating fungal color production in different media.

Table 2 shows the CIELAB color coordinates of the crude pigment extracts obtained from the liquid

**Table 2** CIELAB color coordinates of pigment extracts from liquid media under shaking conditions[a]

| Strain/Media | L | a* | b* | C | $H^0$ |
|---|---|---|---|---|---|
| IBT 7571/M2 | 97.21 | −5.34 | 24.44 | 25.01 | 102.2 |
| IBT 7802/M1 | 66.25 | 9.06 | 75.21 | 75.75 | 83.2 |
| IBT 7802/M3 | 70.37 | 12.11 | 61.81 | 62.98 | 79 |
| IBT 7802/M4 | 70 | 20.93 | 73.57 | 76.48 | 74.2 |
| IBT 41028/M1 | 63.58 | 21.09 | 83.05 | 85.68 | 75.8 |
| IBT 41028/M2 | 92.4 | −0.46 | 41.55 | 41.55 | 90.6 |
| IBT 41028/M3 | 76.11 | 8.49 | 69.71 | 70.22 | 83.1 |
| IBT 41028/M4 | 91.16 | −1.92 | 40.83 | 40.87 | 92.6 |

[a] Average standard deviation for L, a* and b* values shown above was ±0.69 Hue angle ($H^0$) ($h_{ab}$) = $\tan^{-1}$ (b*/a*) chroma (C) = $[(a^*)^2 + (b^*)^2]^{1/2}$

media. The hue angles of the total pigment extracts ranged from 74 to 102 being dark orange to green-yellow. *Epicoccum nigrum* IBT 41028 produced a yellow hue as indicated by their hue angles of 92–93 in M2 and M4 media at the same lightness level. The hue angles changed to the orange side of the CIELAB color space for M3 and M1 media (83 and 76) indicating a higher quantity of yellow pigments also supported by Table 1. This infers that the color components are the same but are produced in higher or lower amounts on changing the media in case of IBT 41028. A markedly different shade of yellow was observed with IBT 7571 in M2 medium as indicated by its hue angle of 102 (Table 2). Different strains of the same species could produce different color hues; an important attribute that possibly be used to get a desired hue by co-cultivating strains of the same species considering the potential biotechnological production of such fungal natural colorants.

Morphologically, all strains grew as pellets and, on one or two occasions, in the form of big floccules. The pellets were described based on their sizes or nature whether smooth or hairy/spiky (Table 1). There were no freely dispersed hyphae or loose mycelial clumps or aggregates observed even though the inocula used was mycelia in the form of small plugs. Morphological variation with respect to the media was observed especially in the type of pellets as pellets with hairy edges (Table 1) comprising of long thread like hyphae were seen typically in the defined M2 media for all the strains where sodium nitrate was used as a nitrogen source. Similar observations were made by Burkholder and Sinnott (1945) for *Epicoccum purpurascens* [*E. purpurascens* is an invalid nomenclatural synonym for *E. nigrum*, Domsch et al. (2007)].

In order to test our hypothesis that the use of buffer can reduce the pellet formation in the liquid media and to see if the resultant morphology has any effect on the pigment production, Bis-tris buffer (pH range 5.8–7.2) was added at 50 mM to M1 and M3 media and it was inoculated with the high yielding strain *E. nigrum* IBT 41028. Pellet formation was diminished in the M3 medium and clusters of dispersed growth were observed. In the M1 medium pellets were reduced to small size irregular floccules with some freely dispersed growth. Pellet size of *Aspergillus niger* was decreased by adding chelating agents EDTA and DTPA (Choudhary and Pirt 1965), but use of Bis-tris buffer for the reduction or depletion of pellets has not yet been reported for *E. nigrum* or any other filamentous fungi to the best of our knowledge. However, inclusion of Bis-tris buffer resulted in a significant decrease in the pigment production in both the media. There was 1.4-fold decrease in the pigment production in case of M1 medium and 1.9 fold reduction in the M3 medium (Fig. 1). Also a noticeable change in the micro-morphology was observed in the buffered and unbuffered M1 and M3 media (Fig. 2a–d). In the buffered M1 medium, the

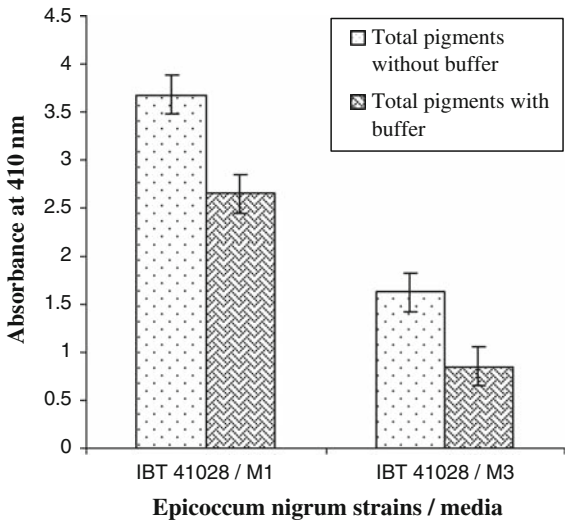
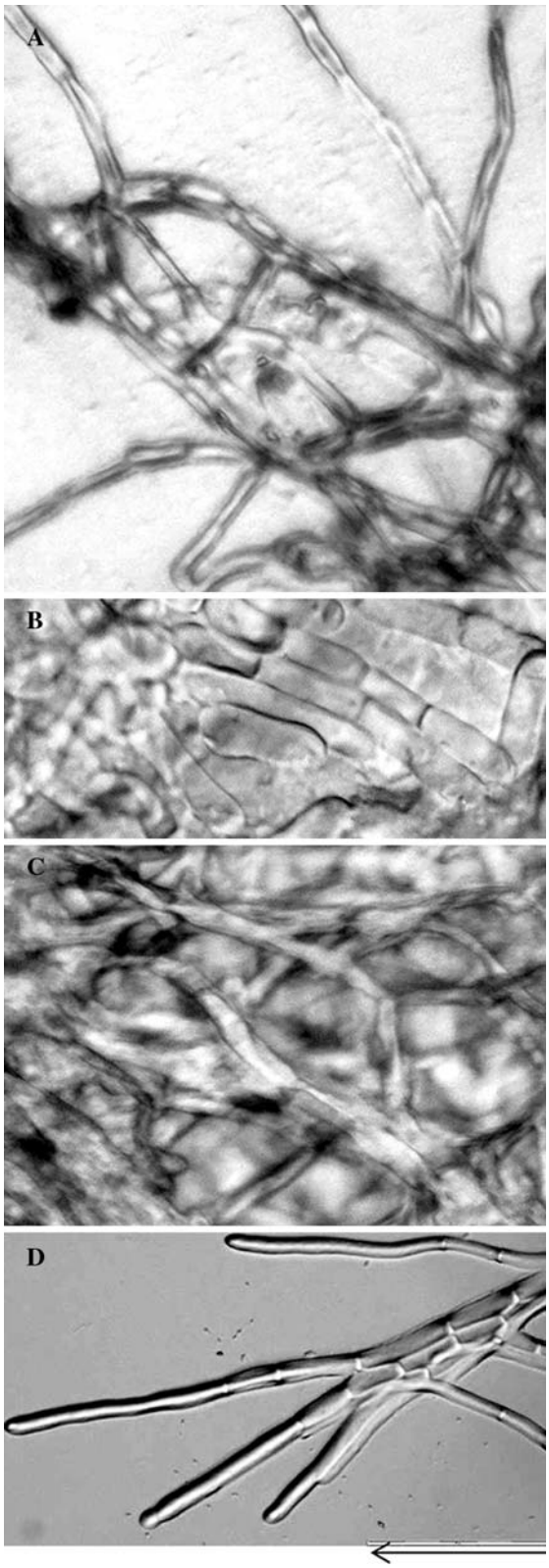



**Fig. 1** Effect of 50 mM Bis-tris buffer on the pigment production by *Epicoccum nigrum* IBT 41028 in M1 and M3 media. Absorbances shown are an average of duplicate determinations with standard deviation




◀**Fig. 2** Effect of 50 mM Bis-tris buffer on the morphology of *Epicoccum nigrum* IBT 41028 in M1 and M3 media. Scale bar (indicated by an arrow) in the right corner of picture D indicates 50 μm and all the pictures were taken under 40× magnification. (**a**) M1 medium without buffer. (**b**) M1 medium with buffer. (**c**) M3 medium without buffer. (**d**) M3 medium with buffer

micro-morphology of the culture was more of wide, thick, shorter hyphae on the periphery of the pellet compared to the buffered M3 media where long, slender, branched hyphae were observed (Fig. 2b, d). Decrease in the pigment production was more pronounced in the buffered M3 medium where the pelleted morphology completely changed to freely dispersed hyphal growth (Fig. 2c, d).

Thus, pelleted or floccular morphology seems to favour the pigment production in case of *E. nigrum* IBT 41028 in the liquid media. This might be explained as cells within the pellet are stressed due to the transportational limitations of nutrients and/or $O_2$, which could result in the possible shift of biosynthetic pathways towards pigment production that are usually involved in the production of other biogenetically related similar secondary metabolites. However, additional work needs to be carried out in order to understand the mode of action of Bis-tris buffer that brought out the morphological changes resulting in the low pigment production.

## Comparative pigment production on rice and in liquid medium by *E. nigrum* IBT 41028

Figure 3 shows the pigment production by *E. nigrum* IBT 41028 on rice and in liquid media with *M. ruber* as control. *Epicoccum nigrum* could produce 6.2-fold higher content of pigments on rice than in the liquid media while *M. ruber* produced 47-fold higher pigments on rice than in the liquid media. Though, the two species preferred rice to the liquid medium, *M. ruber* produced 1.6-fold higher content of pigments on rice than *E. nigrum*. This indicates that the solid support for mycelia facilitates pigment production. A substantially lower volumetric production of *Monascus* pigments in the liquid media compared to on rice or other solid substrate has been reported (Dufosse et al. 2005). To the best of our knowledge, this is the first report on the pigment production by *E. nigrum* on rice or any other solid substrate. One of

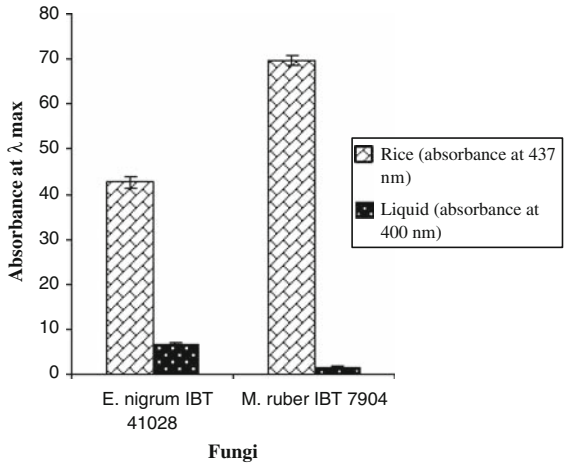



**Fig. 3** Pigment production on rice and in liquid medium by *Epicoccum nigrum* IBT 41028 with *Monascus ruber* IBT 7904 as control. Absorbances shown are an average of duplicate determinations with standard deviation

the important feature of this experiment was the ability of *E. nigrum* to produce 4.6-fold higher pigment contents in liquid, unoptimized fermentation media compared to *M. ruber*. The pigment production was almost twice that in the liquid unoptimized fermentation media than in the initial screening M1 medium. This puts a large incentive to optimize the pigment production by *E. nigrum* in the liquid media as a potential candidate for the large scale industrial production of yellow natural colorants.

The pigment profile of *E. nigrum* IBT 41028 under the two cultivation conditions changed in terms of the number as well as the type of colored components (Table 3). On rice there were two colored components, one each in the neutral/acidic and alkaline elute. The one present in the neutral/acidic elute was identified as the oxopolyketide–orevactaene, previously reported by Shu et al. (1997). While in the liquid media there was only one unidentified colored component present which was not detected on rice. On the other hand, *M. ruber* IBT 7904 produced 10 colored components

**Table 3** Comparative pigment profile of *Epicoccum nigrum* IBT 41028 and *Monascus ruber* 7904 in liquid and on rice based media

| Fungi and media | No. of colored peaks | Retention time (min) | UV–vis absorption wavelength[a] (nm) |
|---|---|---|---|
| Neutral/Acidic elute | | | |
| *M. ruber* IBT 7904 Liquid | nd[b] | | |
| | | 14.47 | 249 (99), 301(100), 376 (74), **421 (82), 526 (86)** |
| | | 15.47 | 253 (82), 297 (100), **421 (91), 526 (91)** |
| | | 19.36 | 231 (94), 288 (20), **390 (100)** |
| | | 19.64 | 222 (100), 284 (57), 310 (57), **425 (52)** |
| | | 19.84 | 222 (73), 288 (47), **385 (100)** |
| | | 20.84 | 218 (65), 293 (34), **469 (100)** |
| | | 21.32 | 213 (78), 240 (84), 288 (48), **416 (98), 447 (99), 469 (100)** |
| | | 22.24 | 222 (100), 288 (84), **482 (94)** |
| | | 22.40 | 249 (98), 288 (98), **429 (100), 522 (100)** |
| *M. ruber* IBT 7904 Rice | 10 | 22.88 | 226 (40), 293 (57), **421 (100)** |
| *E. nigrum* IBT 41028 Liquid | 1 | 21.32 | 231(93), 288 (18), **399 (100)** |
| *E. nigrum* IBT 41028 Rice | 1 | 18.63 | 209 (33), **429 (100)** |
| Alkaline elute | | | |
| *M. ruber* IBT 7904 Liquid | nd[b] | | |
| *M. ruber* IBT 7904 Rice | 1 | 15.66 | 253 (69), 306 (100), **412 (67), 526 (77)** |
| *E. nigrum* IBT 41028 Liquid | nd[b] | | |
| *E. nigrum* IBT 41028 Rice | 1 | 2.78 | 200 (100), 250 (50), **434 (11)** |

[a] Within brackets, % of UV max; in bold VIS region

[b] Not under detectable limit as only major peaks with ≥10% peak area were considered

in the neutral and acidic elute and one component in the alkaline elute when grown on rice (Table 3). No major colored components were detected in the extract obtained from the liquid cultivations. This may be due to the substantially lower pigment production under liquid cultivations compared to on rice by *M. ruber* IBT 7904. Presence of fewer colored components in the extract of *E. nigrum* IBT 41028 can be seen as an added advantage for the natural colorants in order to characterize them with respect to purity and contaminants.

## Conclusion

Among the four tested strains, *E. nigrum* strain IBT 41028 exhibited a broader pigment producing ability in a variety of liquid media, produced 4.6-fold more pigments in liquid medium compared to *M. ruber* and relatively lower pigments on rice but with one or two colored components, a relatively wider color range in the yellow spectrum and thus proved to be worth exploring as a potential candidate for industrial production of natural fungal colorants. In addition, pelleted morphology was shown to have a positive influence on pigment production by *E. nigrum* strain IBT 41028 in liquid media, and the use of Bis-tris buffer was found to reduce the pellet formation.

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## 7.1. Introduction

The possibility of finding more *Monascus*-like pigments from Penicillia belonging to *Penicillium* subgenus *Biverticillium* that would have missed by X-hitting algorithm (**Chapter 5**) either due to the false negatives or due to the non-inclusion of more strains and certain media in that study, was explored. Two more Penicillia were included and the ones that have already been screened were grown on different media. Another issue that is addressed here is whether the pigment producer is able to produce pigments in the liquid medium or not. This is quite a challenge in the future fungal production of pigments to be used as natural colorants. In the light of this, a few chemotaxonomically selected potential pigment producing Penicillia belonging to *Penicillium* subgenus *Biverticillium* were evaluated for their pigment producing ability in the liquid medium. Fungal pigments like most other secondary metabolites are preferably produced on solid substrates as these substrates provide support for the fungal mycelia. The solid state cultivation produces much more pigment compared to submerged cultivation (1). It has also been shown that solid media like rice supports higher pigment yield in case of *Monascus* owing to better hyphal adhesion and penetration offered by the microstructure of the rice grain (2). A unique innovative production method was employed whereby a combination of solid and liquid cultivation technology was used. The fungal mycelia were allowed to grow on a solid support such as Lightweight Expanded Clay Aggregates (LECA). The use of LECA has been described as fungal support matrix by Nielsen *et al*. (3). The LECA were confined to a porous bag like commercially available tea filter and the liquid media was allowed to propagate through the porous bag constantly thereby making a constant supply of nutrients and release of pigments into the media. In addition to the solid support provided by LECA, the advantage in this technique was the easier separation of biomass and the fermentation broth with secreted pigments. Two new chemotaxonomically selected non-pathogenic and non-toxigenic potential pigments producing Penicillia were evaluated for their pigment producing ability in the liquid media with solid support.

## 7.2. Materials and methods

**Pre-selection of fungi, media, and cultivation conditions.** All fungal isolates used in this study were procured from the IBT Culture Collection at Center for Microbial

Biotechnology, Technical University of Denmark, Kgs. Lyngby, Denmark. The fungal isolates were listed by the IBT numbers. All fungi were cultivated on either of the five different solid media *viz*; Yeast extract sucrose (YES) agar; Malt extract agar (MEA), Oatmeal (OAT) agar, Potato dextrose (PD) agar and Czapek-Dox yeast autolysate (CYA) agar (4) or in specific combinations on which maximum pigment was found to be produced with interesting color hues in the red to yellow spectra. The cultures were incubated in the dark at 25 $^{o}$C for 7 days.

Liquid medium consisting of glucose 5% (w/v), $KNO_3$ 1% (w/v), $KH_2PO_4$ 0.8 % (w/v), KCl 0.05 % (w/v), $MgSO_4 \cdot 7H_2O$ 0.05 % (w/v) was used for the cultivations without LECA as solid support. For the liquid media with the solid support, CZ media (4) with 0.5% yeast extract (designated as N1) was used in case of *Penicillium purpurogenum* IBT 11181. N2 medium; where the basal medium was kept the same as in CZ medium (4) except that the carbon sources and nitrogen sources were (g/L): potato starch, 2.75; lactose, 5.5; ammonium nitrate, 1.55; corn steep liquor, 1.55, was used for *Penicillium purpurogenum* IBT 3645. The initial pH of the medium was adjusted to 5.5 using 0.1 M HCl. Approximately 8-9 grams of Light Expanded Clay Aggregates (LECA) were used as solid support retained within a tea filter bag (purchased in a local supermarket) and sterilized by autoclaving. Spores harvested from a week old culture plate (CYA) with a concentration ($3 \times 10^5$ / mL) were inoculated directly onto the LECA contained within the tea filter, which was then transferred into 300 mL baffled Erlenmeyer culture flasks comprising 100 mL of the N1 or N2 media. The experiment was performed in duplicates. The cultures were incubated at 25 $^{0}$C in the dark under shaking conditions (120 rpm).

**Extraction of fungal pigments.** In case of solid media, extraction was carried out by a modified version of the micro-scale extraction method (5), where 6 mm plugs were extracted ultrasonically with solvent containing ethyl acetate, dichloromethane, and methanol in a ratio of 3:2:1 (v/v) with 1% formic acid. The extract was evaporated to dryness in a rotational vacuum concentrator (RVC; Christ Martin, Osterode, Germany). Residue was redissolved in 400 µl methanol, in an ultrasonic bath (Branson 2510, Kell-Strom, Wethersfield, USA) for 10 minutes, and filtered through a 0.45 µl PTFE syringe filter (SRI, Eatontown, NJ, USA). This extract was used for chromatographic analysis.

In case of liquid media with solid support, after 7 days of incubation the tea filter comprising the majority of the fungal biomass adhered to LECA was removed from the

flask and the fermentation broth containing extracellular pigments was filtered through Whatman filter paper # 1 to remove the residual biomass.

**Evaluation of pigment composition from the fermentation broth.** The filtrate was subject to clean-up by solid phase extraction strata-X-C 33 µm cation mixed mode polymer columns (60mg 1ml, Phenomenex, Torrence, CA, USA). 1.2 ml of methanol was used for conditioning followed by 1.2 ml of distilled water for calibration. 1.2 ml of samples, acidified with 0.1 % phosphoric acid (1: 6 v/v) were loaded in a vacuum manifold, and washed with 0.1% phosphoric acid. Elution of the pigment mixture, bound in the matrix of the cartridges, was carried out using methanol which could elute neutral and acidic components. The pigment extract so obtained was subjected to high resolution LC-DAD-MS analysis.

**Chromatographic analysis.** High-resolution LC-DAD-MS was performed on an Agilent HP 1100 liquid chromatograph (LC) system with a photodiode array detector (DAD) and a 50 × 2 mm i.d., 3 µm, Luna C 18 II column (Phenomenex, Torrance, CA). The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, U.K.) with a Z-spray electrospray ionization (ESI) source, and a LockSpray probe and controlled by the MassLynx 4.0 software. MS system was operated in the positive ESI mode using a water-acetonitrile gradient system starting from 15% acetonitrile, which was increased linearly to 100% in 20 minutes with a holding time of 5 minutes. The water was buffered with 10 mM ammonium formate and 20 mM formic acid and the acetonitrile with 20 mM formic acid. The instrument was tuned to a resolution > 7000 (at half peak height). The method is well established at our research center for the metabolite profiling of filamentous fungi grown on solid media. It is described by Nielsen *et al*. (6).

For the extracellular pigments extracted from the liquid media, the solvent system used was water with 0.1% formic acid and acetonitrile with 0.1% formic acid. The gradient started from 5% acetonitrile and increased to 100% in 20 minutes and was hold at 100% for 2 minutes. The MS conditions were the same as mentioned earlier.

**Analysis of LC-DAD-MS Data.** The colored components in the pigment extract were detected in the UV-vis chromatogram of 390-700 nm. The identification of N-

glutarylmonascorubramine and N-glutarylrubropunctamine was based on both UV-vis and mass spectra from total ion chromatogram (m/z 100-900) from positive ion electro spray. The UV-vis spectrum was obtained after background subtraction. The DAD-MS data for N-glutarylmonascorubramine and N-glutarylrubropunctamine is shown below:

N-glutarylmonascorubramine was detected as *m/z* 512.23 $[M + H]^+$ and confirmed by the adducts *m/z* 534.22 $[M + Na]^+$ and *m/z* 575.26 $[M + Na + CH_3CN]^+$. The UV-vis spectrum was $\lambda_{max}$: 212, with a shoulder at 272, 420 and 504.

N-glutarylrubropunctamine was detected as *m/z* 484.20 $[M + H]^+$ and confirmed by the adducts *m/z* 506.21 $[M + Na]^+$ and *m/z* 547.22 $[M + Na + CH_3CN]^+$. The UV-vis spectrum was $\lambda_{max}$: 200, 250, 274, 430 and 522.

## 7.3 Results

**Table 7.1** brings out 5 strains as potentially safe novel producers of *Monascus*-like azaphilone polyketide based pigments such as ankaflavin, monascin, monascorubrin, monascorubramine, and PP-R (**Figure 7.1**) on solid media, in addition to the 3 strains reported previously (7, 8). Notably four out of the five strains tested (**Table 7.2**), have been found to produce extracellular pigments in the liquid media with or without solid support. The pigment producing ability of these four Penicillia in the liquid medium was found to be better than the tested *Monascus ruber* strains used as controls. Two of the Penicillia *viz. P. purpurogenum* IBT 11181 and *P. purpurogenum* IBT 3645 were tested for their pigment producing ability in the liquid medium with LECA as solid support for mycelia. For the first time, N-glutarylmonascorubramine and N-glutarylrubropunctamine (**Figure 7.1**); the two known water-soluble *Monascus*-pigments were discovered in the extracellular pigment extract obtained from the liquid medium (N1) of *P. purpurogenum* IBT 11181 (**Figure 7.2**). The identity was based on the UV-vis and mass spectra (**Figure 7.2**). N-glutarylmonascorubramine was also discovered in the extracellular pigment extract of *P. purpurogenum* IBT 3645 obtained from the liquid medium (N2) as shown in (**Figure 7.3**).

**Table 7.1** Potentially safe novel producers of *Monascus*-like azaphilone polyketide based pigments on solid media

| Fungal Name/Serial No. | IBT Culture Collection No. | Other culture collection No. | Source of isolation | Pigments identified | Known mycotoxins |
|---|---|---|---|---|---|
| *Penicillium purpurogenum* **1**. | 21347 | - | Human saliva | .[1]PP-R. | None |
| **2**. | 23082 | RMF 81.01 | Soil | [2]PP-R. | None |
| **3**. | 3967 | NRRL 1147 | Unknown | [3]Monascorubramine, Monascin. | None |
| *Penicillium aculeatum* **4.** | 14259 | NRRL 2129 | Weathering fabric | [1]Monascorubrin | None |
| *Penicillium funiculosum* **5**. | 3954 | NRRL 2119 | Unknown | [4]Ankaflavin. | None |

[1]on CYA agar, [2]on YES Agar, [3]on OAT agar, [4] on MEA agar,

**Table 7.2.** Pigment producing ability and production pattern on solid and in liquid media with or without LECA as solid support of some representative non-toxigenic and non-pathogenic Penicillia with *Monascus ruber* as control.

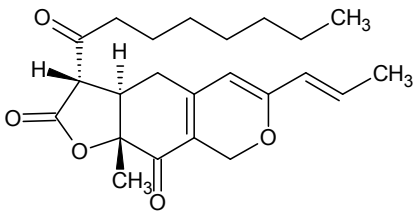
| Fungal Identity | | | Best solid media for pigment production | Pigment production pattern[1] | Pigment producing ability[2] in liquid medium[3] | |
|---|---|---|---|---|---|---|
| **Genus** | **Species** | **IBT No.** | | | **Stationary** | **Shaking** |
| *Monascus* | *ruber* | 98585 | MEA | M | ++ | + |
| *Monascus* | *ruber* | 7904 | OAT,MEA | M | - | + |
| *Penicillium* | *funiculosum* | 3954 | MEA | E | - | - |
| *Penicillium* | *purpurogenum* | 11180 | CYA | E | +++ | ++++ |
| *Penicillium* | *purpurogenum* | 3967 | YES, CYA | E | ++++ | +++ |
| **Penicillium* | *purpurogenum* | 11181 | YES, CYA | E | ++ | ++++ |
| **Penicillium* | *purpurogenum* | 3645 | YES, CYA | E | ++ | ++++ |

[1]'E' refers to 'Extracellular', while 'M' refers to 'Mycelial' pigments as observed on the solid media.

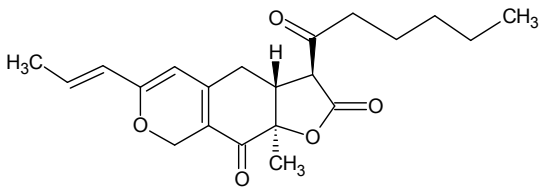
[2]pigment producing ability was qualitatively assessed and rated on a scale of + to +++++ for the mild pigmentation to intense pigmentation with an intermediate level represented by +++. '-'indicates absence of pigment production. Experiment was performed in duplicates. [3] Liquid medium consisting of glucose 5% (w/v), $KNO_3$ 1% (w/v), $KH_2PO_4$ 0.8 % (w/v), KCl 0.05 % (w/v), $MgSO_4 \cdot 7H_2O$ 0.05 % (w/v) was used, * grown in liquid medium with LECA as solid support and shown to produce extracellular *Monascus* pigments (See text)

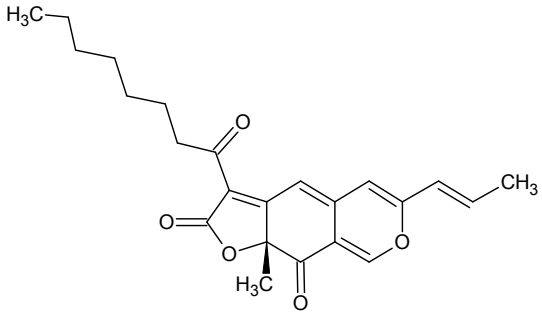
**Figure 7.1** Structures of *Monascus* pigments detected and identified in the present work.

***Ankaflavin ($C_{23}H_{30}O_5$, 386.48)***

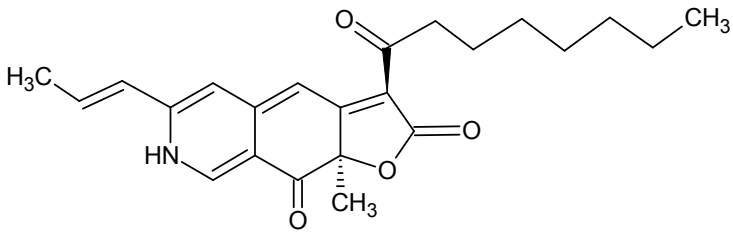


***Monascin ($C_{21}H_{26}O_5$,358.43)***

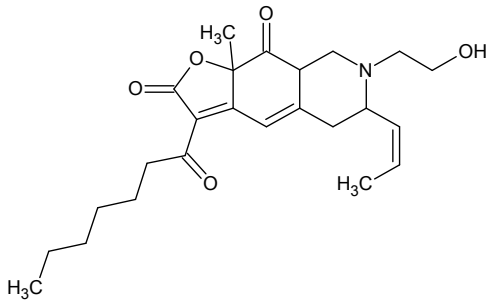




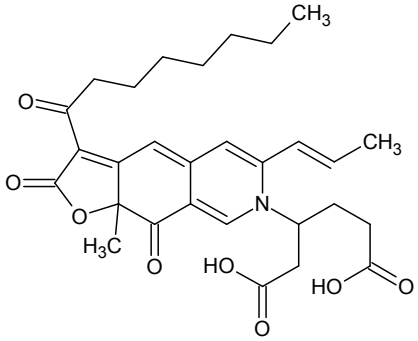
Monascorubrin ($C_{23}H_{26}O_5$, 382.45)



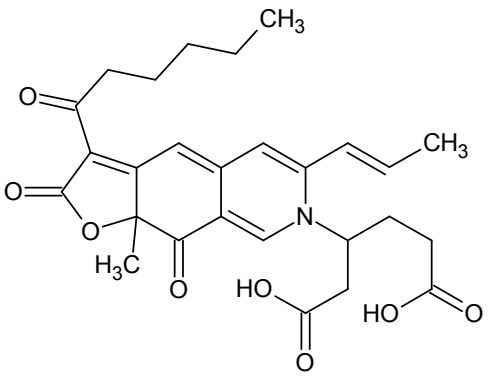
Monascorubramine ($C_{23}H_{27}NO_4$, 381.46)



PP-R; 7-(2-Hydroxyethyl)-monascorubramine ($C_{25}H_{31}NO_5$, 426.23)



N-glutarylmonascorubramine ($C_{28}H_{33}NO_8$, 511.23)



N-glutarylrubropunctamine ($C_{26}H_{29}NO_8$, 483.20)

**Figure 7.2. Presence of N-glutarylmonascorubramine and N-glutarylrubropunctamine in the extracellular pigment extract of *Penicillium purpurogenum* IBT 11181.**

The strain was grown in N1 liquid medium with LECA as a solid support. Total ion chromatogram (m/z 100-900), on X-axis time is shown, and on Y-axis % of the ion count (**A**), and UV-vis chromatogram at 390-700 nm, on X-axis time is shown, and on Y-axis % of the sum of the absorbencies, a relative value (**B**), mass spectrum (**A1**) and UV-vis spectrum of N-glutarylmonascorubramine (**B1**), mass spectrum (**A2**) and UV-vis spectrum of N-glutarylrubropunctamine (**B2**).

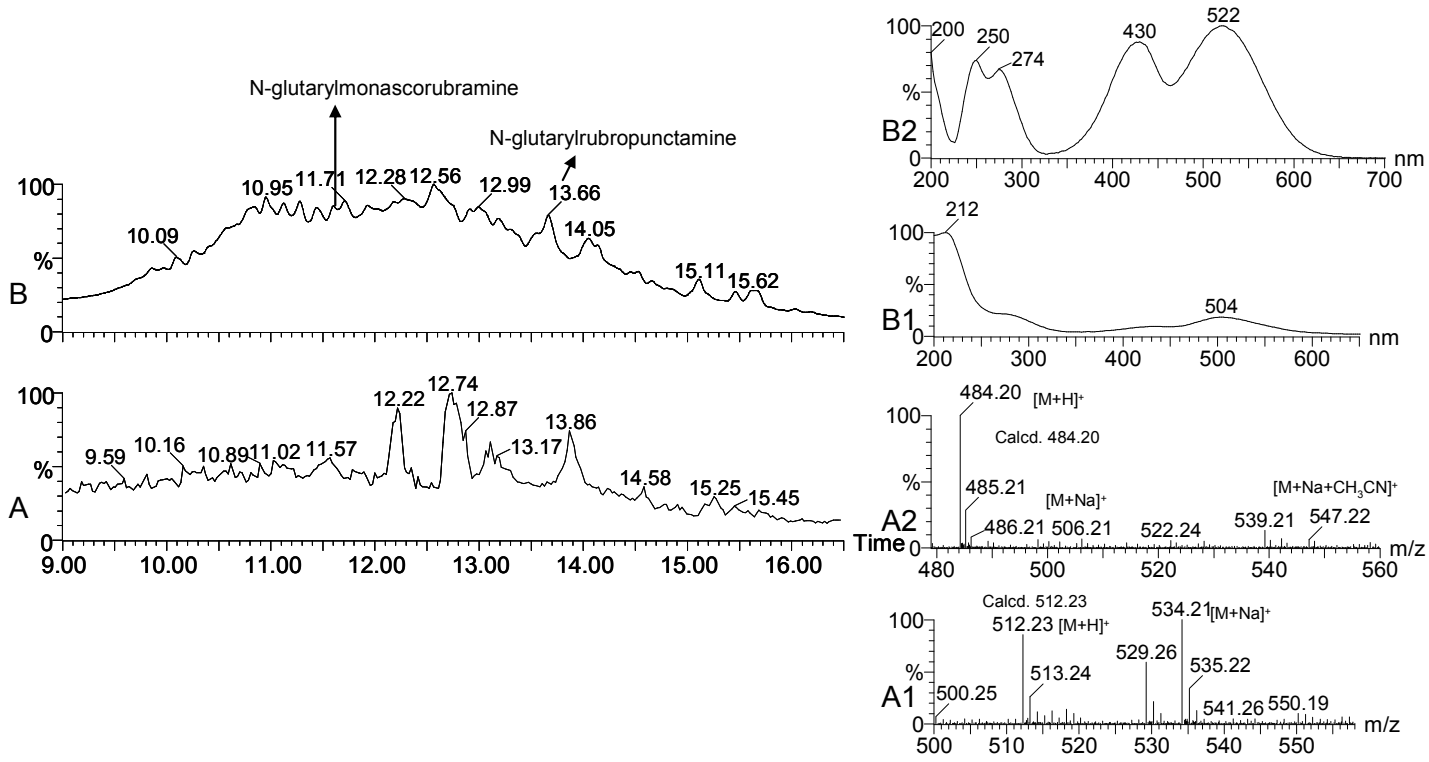
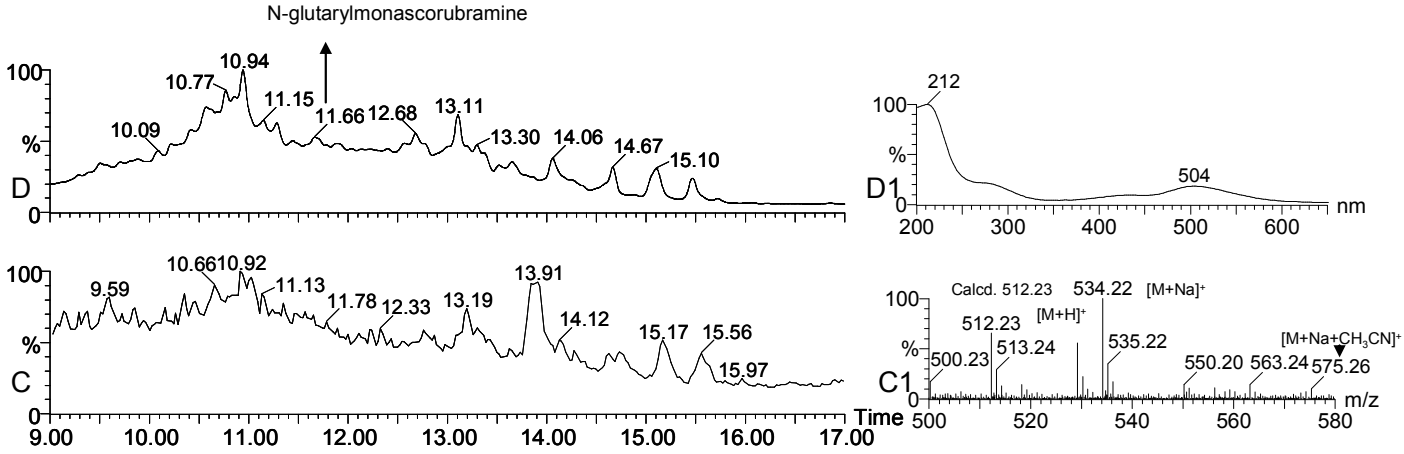


**Figure 7.3 Presence of N-glutarylmonascorubramine in the extracellular pigment extract of *Penicillium purpurogenum* IBT 3645.**

The strain was grown in N2 liquid medium with LECA as a solid support. Total ion chromatogram (m/z 100-900), on X-axis time is shown, and on Y-axis % of the ion count (**C**), and UV-vis chromatogram at 390-700 nm, on X-axis time is shown, and on Y-axis % of the sum of the absorbencies, a relative value (**D**), mass spectrum (**C1**) and UV-vis spectrum of N-glutarylmonascorubramine (**D1**).




## 7.4 Discussion

Several of the already known *Monascus*-like pigments including two water-soluble extracellular pigments (**Table 7.1, Figure 7.1, 7.2, 7.3**) were identified in *Penicillium* species that do not produce any known mycotoxins when grown under the laboratory conditions used in this study. The production of extracellular pigments by some of the tested cell factories in the liquid media using a solid support has added value to these cell factories as future production strains. N-glutarylmonascorubramine and N-glutarylrubropunctamine have been reported as water-soluble extracellular pigments of *Monascus ruber* (9, 10, 11). The identification of N-glutarylmonascorubramine was based on mass and UV-vis spectra that matched quite well with the previous reports (9, 11). The mass spectra of N-glutarylrubropunctamine matched very well with the one reported by Hajjaj *et al.* (11). The UV-vis spectrum of N-glutarylrubropunctamine was very similar to that of N-glutarylmonascorubramine as the two compounds differed only in their aliphatic side chain. Multiple extracellular pigments (**Figure 7.2 & 7.3**) were formed from a complex nitrogen source such as yeast extract (as in case of N1 medium) or a combination

of such complex nitrogen sources as corn steep liquor and yeast extract (as in case of N2 medium) and rubropunctatin and/or monascorubrin in the media possibly by Schiff base formation type of reaction mechanism. This mechanism was reported previously (9, 11) for the formation of these water soluble pigments from their counter parts which are aminophilic in nature being azaphilones. Hajjaj *et al.* (11) reported the formation of 4 pigment molecules that constituted 91% of the pigment production when only one nitrogen source (monosodium glutamate) in the medium was used with 20 g/L glucose. Our results indicate formation of much more than 4 pigment components for both of the tested Penicillia (**Figure 7.2 & 7.3**) possibly due to the availability of several amino acids from the complex nitrogen source/s provided in the media. Thus, in the future it is possible to obtain desired number of pigment components by using defined nitrogen sources that would provide specific amino acid/s moiety to be incorporated into the azaphilone pigments to form water soluble extracellular pigments. As these water soluble extracellular pigments are more photostable than their orange counterparts (12), this is very significant for the biotechnological production of such natural colorants in these newly discovered cell factories Thus it can be inferred that the discovery of such potentially safe cell factories for polyketide natural food colorants using chemotaxonomic approach is a landmark that would redefine the biotechnological production of food colorants derived from such an agro-independent source of colorants as filamentous fungi. Moreover, this also can tackle the current issue of restricted use of *Monascus* pigments due to the co-production of toxic metabolites.

## 7.5 References

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## 7.6 Special research note on preliminary study on media optimization and design for "tailor-made" orange-red colorants

### 7.6.1. Aim

To demonstrate that the supply of various carbon and/or nitrogen sources modulate different pigment production by *P. purpurogenum* IBT 11181 resulting in "tailor-made" orange-red colorants.

### 7.6.2 Materials and Methods

**Media and cultivation conditions.**For the liquid media with the solid support, the basal medium was as follows (g/L): Yeast extract: 5; $K_2HPO_4$ : 1; $MgSO_4.7H_2O$: 0.5; $FeSO_4.7H_2O$: 0.01; KCl: 0.5. The carbon and nitrogen sources were: potato starch (PS), lactose (L), ammonium nitrate(AN), and corn steep liquor (CSL). The combination was obtained by the software MODDE for screening using fractional factorial design as shown in **Table 7.3**. The initial pH of the medium was adjusted to 5.5 using 0.1 M HCl. 8-9 3.25 ± 0.19 g of Light Expanded Clay Aggregates (LECA) were used as solid support retained within a tea filter bag (purchased in a local supermarket) and sterilized by autoclaving. Spores harvested from a week old culture plate (CYA) with a concentration ($3 \times 10^5$ / mL) were inoculated directly onto the LECA contained within the tea filter, which was then transferred into 300 mL baffled Erlenmeyer culture flasks comprising 100 mL of the media. The experiment was performed in duplicates. The cultures were incubated at 25 $^0$C in the dark under shaking conditions (120 rpm) for 10 days.

**Analysis of pigments by colorimetry.** Filteration of the fermentation broth was carried out using Whatman filter paper # 1 to remove the mycelial debris and/or spores. The filtrate so obtained was diluted so as to bring the absorbencies in the linear range of Beer-Lambert's law, and the absorbencies were recorded at their absorption maxima by the spectrophotometer (Shimadzu Model UV-160). A characteristic two absorption peaks were seen in the visible region of spectra. The first one at around 495 nm and the other one ranged from 407-420 nm. The ratio of the two absorbencies was used to determine the red color index of the pigments, i.e. a higher value of the ratio represents a higher proportion of the red pigments.

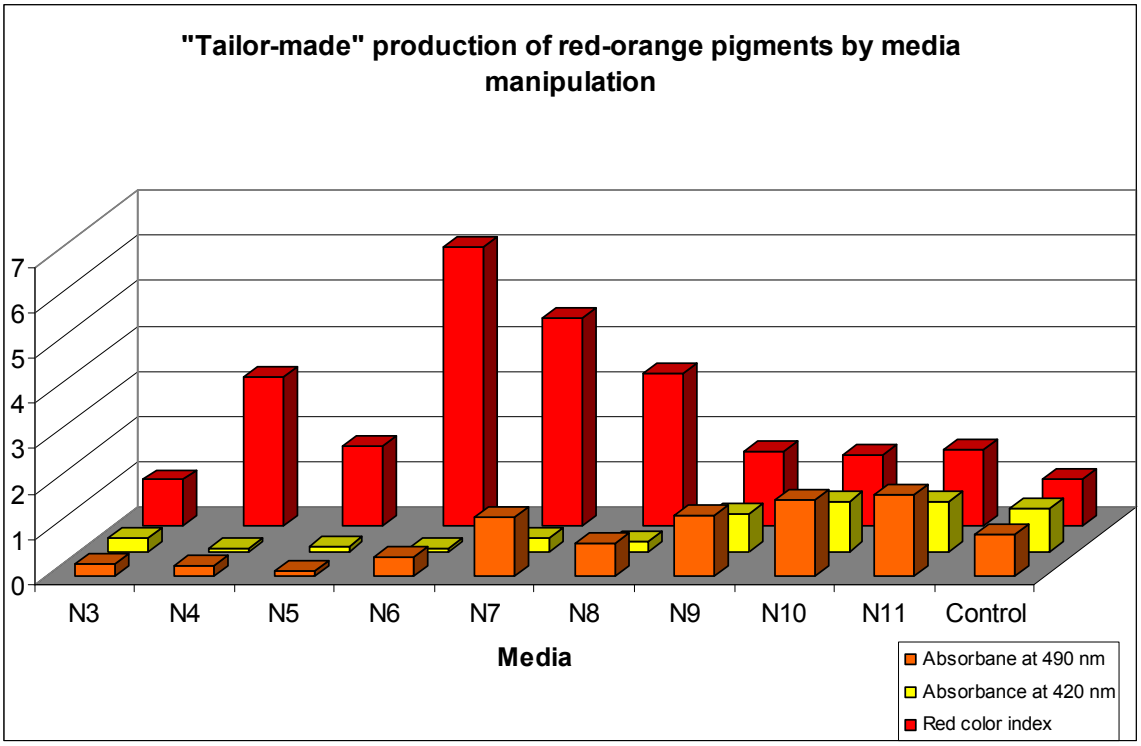
### 7.6.3 Results and Discussion

The results (**Figure 7. 4)** indicate that the medium N11 yielded maximum pigment production in the present experimental set up closely followed by medium N10. The color hue was almost the same in both of the media as indicated by a similar red color index. It was also possible to get the various hues of red-orange by changing the combination of carbon and/or nitrogen sources as it can be seen from the increasing or decreasing red color index (N6, N7, and N8 in **Table 7.3**).

**Table 7.3.** Media formulation scheme. Control was CZ medium with 0.5% yeast extract

| **Expt/ Media** | **Concentration (g/L)** | | | |
|---|---|---|---|---|
| | **AN** | **PS** | **L** | **CSL** |
| N3 | 0.1 | 0.5 | 1 | 0.1 |
| N4 | 0.1 | 5 | 1 | 0.1 |
| N5 | 3 | 0.5 | 10 | 0.1 |
| N6 | 0.1 | 5 | 10 | 0.1 |
| N7 | 3 | 0.5 | 10 | 3 |
| N8 | 0.1 | 2.75 | 5.5 | 1.55 |
| N9 | 1.55 | 2.75 | 1 | 1.55 |
| N10 | 1.55 | 2.75 | 5.5 | 0.1 |
| Center Point Replicate | | | | |
| N11 | 1.55 | 2.75 | 5.5 | 1.55 |
| Control | - | - | - | - |

**Figure 7.4.** Effect of various carbon ad nitrogen sources on pigment production by *P. purpurogenum* IBT 11181 in liquid medium using LECA as solid support




The red pigment production was increased in N4 medium as compared to N3 medium when potato starch concentration increased from 0.5 g/L to 5g/L keeping the other ingredients same. Comparing N5 and N7, an increase in the concentration of CSL from 0.1 to 3 g/L keeping the other ingredients unchanged in the medium N7 resulted in not only the higher yield but also a relatively more red color hue. This implies that manipulation of carbon and/or nitrogen sources can be of high impact in designing the tailor-made colorants.

### 7.6.4 Conclusion

Different carbon and nitrogen sources have been shown to induce production of different pigments resulting in a different color hue. A systematic evaluation of the effect of different media components on pigment production – and/or a better understanding of the factors inducing pigment production in fungi – is currently highly warranted in order to optimize the pigment production.

## Conclusions of the work presented in the thesis

- The pertinent use of chemotaxonomic trait of metabolite profiling using tools such as HPLC-DAD-MS and *a priori* knowledge of fungal extrolites is a rational approach towards selection of potentially safe polyketide natural colorant producing fungal cell factories that are neither known to be human pathogens nor to produce any known mycotoxins. The present study is a proof of the above-mentioned concept.
- The present study also brought out the potentially safe future cell factory possibilities of filamentous fungi that produce polyketide pigments naturally without the use of genetic manipulation techniques.
- It has been demonstrated that filamentous fungi can produce known or novel polyketide colorants as lead compounds with a potential of improved functionality in food model systems.
- Different carbon and nitrogen sources have been shown to induce production of different pigments resulting in a different color hue giving rise to the possibility of producing “tailor-made” colorants.

## Future Perspectives

- A systematic evaluation of the effect of different media components on pigment production – and/or a better understanding of the factors inducing pigment production in fungi – is currently highly warranted in order not only to optimize but also for the robust pigment production.
- The issue of toxicity (especially for the food use) of the pigment extracts is not addressed in this thesis (even though potentially safe pigment producers were discovered), and therefore it forms the future perspective of the present study. It is worthwhile to mention that the pigment extracts contained *Monascus* pigments and/or their derivatives that have been consumed over hundreds of years without an acute case of health hazard meaning that they must not be toxic on their own if used in an appropriate dosage.

# APPENDIX A

Manuscript based on Chapter 3 and a part of Chapter 7.

**Identification of potentially safe promising fungal cell factories for the production of polyketide natural food colorants using chemotaxonomic rationale.**

Sameer AS Mapari, Anne S. Meyer, Ulf Thrane, and Jens C. Frisvad.
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# Identification of potentially safe promising fungal cell factories for the production of polyketide natural food colorants using chemotaxonomic rationale

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## Abstract

**Background:** Colorants derived from natural sources look set to overtake synthetic colorants in market value as manufacturers continue to meet the rising demand for clean label ingredients – particularly in food applications. Many ascomycetous fungi naturally synthesize and secrete pigments and thus provide readily available additional and/or alternative sources of natural colorants that are independent of agro-climatic conditions. With an appropriately selected fungus; using in particular chemotaxonomy as a guide, the fungal natural colorants could be produced in high yields by using the optimized cultivation technology. This approach could secure efficient production of pigments avoiding use of genetic manipulation.

**Results:** Polyketide pigment producing ascomycetous fungi were evaluated for their potential as production organisms based on *a priori* knowledge on species-specific pigment and potential mycotoxin production and BioSafety level (BSL) classification. Based on taxonomic knowledge, we pre-selected ascomycetous fungi belonging to *Penicillium* subgenus *Biverticillium* that produced yellow, orange or red pigments while deselecting *Penicillium marneffei*; a well known human pathogen in addition to other mycotoxigenic fungi belonging to the same group. We identified 10 strains belonging to 4 species; *viz*. *P. purpurogenum, P. aculeatum, P. funiculosum*, and *P. pinophilum* as potential pigment producers that produced *Monascus*-like pigments but no known mycotoxins. The selection/deselection protocol was illustrated in the pigment extracts of *P. aculeatum* IBT 14259 and *P. crateriforme* IBT 5015 analysed by HPLC-DAD-MS. In addition, extracellular pigment producing ability of some of the potential pigment producers was evaluated in liquid media with a solid support and N-glutarylmonascorubramine was discovered in the partially purified pigment extract of *P. purpurogenum* IBT 11181 and IBT 3645.

**Conclusion:** The present work brought out that the use of chemotaxonomic tools and *a priori* knowledge of fungal extrolites is a rational approach towards selection of fungal polyketide pigment producers considering the enormous chemical diversity and biodiversity of ascomycetous fungi. This rationale could be very handy for the selection of potentially safe fungal cell factories not only for polyketide pigments but also for the other industrially important polyketides; the molecular and genetic basis for the biosynthesis of which has not yet been examined in detail. In addition, 4 out of the 10 chemotaxonomically selected promising *Penicillium* strains were shown to produce extracellular pigments in the liquid media using a solid support indicating future cell factory possibilities for polyketide natural food colorants.

## Background

Due to the global increase in the manufacture of processed foods and the continued consumer demands for natural food ingredients, the market for natural colorants for food use is estimated to grow [1]. Currently, the vast majority of the natural food colorants permitted in the European Union and the United States are derived by extraction of the pigments from raw materials obtained from the flowering-plants of the kingdom Plantae [2]. The production of many existing natural colorants of plant origin has a disadvantage of dependence on the supply of raw materials, which are influenced by agro-climatic conditions – in addition, their chemical profile may vary from batch-to-batch. Moreover, many of the pigments derived from the contemporary sources are sensitive to heat, light, and oxygen, and some may even change their colour in response to pH changes as in case of anthocyanins [3]. Many ascomycetous fungi naturally synthesize and secrete pigments and may thus provide a more reliable source for natural, "organic" food colorants with improved functionalities [4]. The diversity of fungal pigments is not only found in their chemical structures but also in the colour range of these pigments that may add new or additional hues to the colour palette of the existing colorants derived from contemporary sources [5].

With an appropriately selected fungus the fungal natural colorants; unlike flowering plants, plant cell or algal sources of colorants, could be produced in high yields by using the available cultivation technology without potential genetic manipulation as tougher legislation and sceptical attitude of consumers make it rather difficult for the acceptance of genetically modified food. The controlled cultivation of pigment producing fungi in bioreactors has the potential to compete with any other means of production and can potentially provide unlimited quantities of colourings provided that imperative toxicological studies are carried out. However, a first requirement is that the potential fungus producing the pigment(s) is non-toxigenic under the given broader range of production conditions and is non-pathogenic to humans. The ability of filamentous fungi to co-produce mycotoxins along with industrially useful extrolites, e.g. as in case of citrinin produced by some of the pigment and statin producing *Monascus* species [6], is a major bottleneck in their approval by the legislative authorities. Some of the pigment producers, for instance, *Penicillium marneffei*, could even be human pathogens [7]. Different cultivation media have been shown to induce production of different pigments [5]; a systematic evaluation of the effect of different media components on pigment production – and/or a better understanding of the factors inducing pigment production in fungi – is currently highly warranted in order to optimize the pigment production.

Another challenge in the fungal production of pigments to be used as natural colorants is whether the pigment producer is able to produce pigments in the liquid media or not. Fungal pigments like most other secondary metabolites are preferably produced on solid substrates as these substrates provide support for the fungal mycelia. Therefore, we employed a combination of solid and liquid cultivation technology whereby the fungal mycelia were allowed to grow on a solid support like Lightweight Expanded Clay Aggregates (LECA). The use of LECA has been described as fungal support matrix by Nielsen et al. [8]. Another advantage in this technique was the easier separation of biomass and the fermentation broth with secreted pigments.

Thus, it is of utmost importance to address the question of how to rationally select a promising fungal pigment producer – meeting the above requirements – considering the enormous chemical and biodiversity of fungi. Moreover, the molecular and genetic basis for the polyketide pigment biosynthesis in fungi have not yet been examined in detail leaving genomic approaches for screening unfeasible at this point of time.

In the light of this, we provide a comprehensive list, based on chemotaxonomy, of a majority of the polyketide pigment producing ascomycetous fungi, their pigment composition, and the toxigenic potential with a list of known coloured as well as uncoloured toxic metabolites. Based on taxonomic knowledge, we focussed on pigment producing ascomycetous fungi belonging to *Penicillium* subgenus *Biverticillium* that produced yellow-orange-red pigments while human pathogenic and mycotoxigenic strains belonging to the same group were deselected. We exemplify our chemotaxonomic selection and/or de-selection approach in two Penicillia *viz. Penicillium aculeatum* and *Penicillium crateriforme* grown on 5 different complex solid media to identify potential pigment producers that produced known pigments with or without mycotoxin. The aim of this study was to prove the pertinent use of chemotaxonomic trait of metabolite profiling by powerful tools as HPLC-DAD-MS to come up with promising polyketide pigment producing cell factories that are neither known to be human pathogens nor to produce any known mycotoxins. The ultimate goal is to establish such potentially safe fungal cell factories for the production of polyketide natural colorants. In addition, we also evaluated 4 potential pigment producers for their pigment producing ability in the liquid media using solid support.

## Results

### *Chemotaxonomic selection/de-selection for potential polyketide pigment producing ascomycetous fungi*

Many species of *Penicillium* and *Aspergillus* and their teleomorphs have been metabolically profiled for production

of pigments and mycotoxins at our research center. The major pigment profiles are listed in Table 1 with citations to relevant literature at the species level [4,5,9-23]. In addition to the well-known pigment producing *Monascus* species used in Asia many species of *Fusarium*, *Alternaria*, and *Epicoccum* are also polyketide pigment producers, and strains from these genera have also been profiled at our research center. Detailed profiles are listed in Table 2 with relevant citations at the species level [5,24-28]. To illustrate the diversity and the potential of possible pigments producers Table 2 also gives citations to pigment production by *Cladosporium*, *Cordyceps*, *Curvularia*, *Drechslera* and *Paecilomyces* species [4,29-31]. This *a priori* knowledge on potential mycotoxin production and the evaluation of pathogenic potential on the basis of BioSafety Level (BSL) classification [32] formed the basis of our pre-selection/deselection of pigment producers. Strains of the species belonging to *Penicillium* subgenus *Biverticillium* (Table 1) were known to produce copious amounts of yellow, orange and red pigments on solid media; one of the representative red pigment producers is shown in Figure 1. These strains of the species were chosen to be studied. We deselected strains of the 4 species *viz. P. islandicum, P. marneffei, P. variabile* and *P. rugulosum* including the teleomorph *Talaromyces macrosporus* being either pathogenic and/or mycotoxigenic. We screened 11 strains belonging to the rest of the 5 species *viz. P. purpurogenum, P. crateriforme, P. aculeatum, P. funiculosum*, and *P. pinophilum* where by *P. crateriforme* served as a positive control in which the presence of mycotoxin was known *a priori* but the pigment was still uncharacterized. The chromatographic analyses of two representative pigment extracts that illustrate our selection/de-selection approach are presented in Figure 2, Figure 3 and Figure 4. Figure 2 depicts the extracted ion chromatogram (m/z 269.12) obtained by positive ESI chromatography using authentic standard of rugulovasine A and B. The pigment extracts of *Penicillium crateriforme* IBT 5015 grown on CYA (Figure 2B), MEA (Figure 2C), PD (Figure 2D), OAT (Figure 2E), and YES (Figure 2F) media were found to be positive for the presence of rugulovasine A and B. Figure 3 depicts the absence of rugulovasine A and B in the pigment extracts of *Penicillium aculeatum* IBT 14259 grown on YES (Figure 3B), PD (Figure 3C), OAT (Figure 3D), MEA (Figure 3E), and CYA (Figure 3F). Both of these Penicillia were found to produce a well-known orange *Monascus* pigment, monascorubrin, in CYA as shown in Figure 4B and Figure 4C by means of the extracted ion chromatogram (m/z 383.19) and mass spectrum, obtained by positive ESI chromatography using authentic standard. Since *Penicillium crateriforme* IBT 5015 produced rugulovasine A and B, in addition to rubratoxin, and spiculisporic acid [9], it was deselected, while *Penicillium aculeatum* IBT 14259 was selected. The rest of the 10 strains (Table 3) were found to produce known *Monascus*-like azaphilone pigments but none of the known mycotoxins such as rubratoxin, luteoskyrin, islanditoxin, rugulosin, cyclochlorotine, erythroskyrin, emodin, spiculisporic acid, and rugulovasine A and B associated with this group of fungi [9]. To the best of our knowledge, the genes or the enzymes involved in the biosynthesis of the above-mentioned mycotoxins are not yet characterized.

### *New potentially safe fungal cell factories for the production of* Monascus*-like pigments*

Table 3 brings out 7 strains (2, 3, 4, 5, 6, 7 and 9 in Table 3) as novel producers of *Monascus*-like azaphilone polyketide based pigments such as ankaflavin, monascin, monascorubrin, monascorubramine, PP-R, N-glutarylmonascorubramine, and N-glutarylrubropunctamine (Figure 5), in addition to the 3 strains (1, 8 and 10 in Table 3) reported previously [5,33]. None of these strains were found to produce any known mycotoxins in the mentioned media and under the lab conditions of pigment production. Notably four of the strains tested so far (Table 3), have been found to produce extracellular pigments in the liquid media with LECA as a solid support. For the first time, N-glutarylmonascorubramine and N-glutarylrubropunctamine; the two known water-soluble *Monascus*-pigments were discovered in the extracellular pigment extract obtained from the liquid medium (N1) of *P. purpurogenum* IBT 11181 (Figure 6). The identity was based on the UV-vis and mass spectra (Figure 6). N-glutarylmonascorubramine was also discovered in the extracellular pigment extract of *P. purpurogenum* IBT 3645 obtained from the liquid medium (N2) as shown in Figure 7.

## Discussion

### *Chemotaxonomic selection/de-selection for potential polyketide pigment producing ascomycetous fungi*

The strains of the species in Table 1 and Table 2 are yet to be investigated as potential production strains on different solid and in liquid media. Some of them may be promising as potentially safe cell factories for pigments with an array of different colors indicating that a lot more fungal biodiversity is yet to be explored for the discovery of novel sources of natural colorants. *Epicoccum nigrum* (Table 2) has been evaluated for pigment production [34], and *Penicillium herquei* (Table 1) has been partially evaluated (data unpublished). They could be potential cell factories for natural green-yellow to yellow colorants. Biosynthetically, a majority of pigments produced by filamentous fungi are polyketide-based (some may involve polyketide-amino acid mixed biosynthesis) and involve complex pathways catalysed by iterative type I polyketide synthases as exemplified in case of pigments produced by *Monascus ruber* [35]. A complete knowledge about the biosynthetic pathway of polyketide pigments including the extensively studied *Monascus* pigments is not yet available.

**Table 1: Comprehensive list of polyketide pigment producing *Penicillium* and *Aspergillus* species and their known teleomorphs.**

| Fungal species | Pigment composition (colour). Toxic colored compounds in bold | Major known mycotoxic uncolored metabolites | BioSafety Level (BSL) classification[1][32] | Comment[2] | Ref. |
|---|---|---|---|---|---|
| ***Penicillium* subgenus *Penicillium*** | | | | | |
| *P. atramentosum* | Uncharacterized dark brown | Roquefortine C<br>Rugulovasine A & B | unknown | No | [10] |
| *P. atrosanguineum* | Phoenicin (red)<br>Uncharacterized yellow and red | unknown | unknown | TBI | [11] |
| *P. atrovenetum* | Atrovenetin (yellow)<br>Norherqueinone (red) | beta-Nitropropionic acid | unknown | No | [12] |
| *P. aurantiogriseum* | Uncharacterized | Nephrotoxic glycopeptides<br>Penicillic acid<br>Verrucosidin | I | No | [10] |
| *P. brevicompactum* | Xanthoepocin (yellow) | Botryodiploidin<br>Mycophenolic acid | I | No | [10] |
| *P. chrysogenum* | Sorbicillins (yellow)<br>Xanthocillins (yellow) | Roquefortine C | I | No | [10] |
| *P. citrinum* | Anthraquinones (yellow)<br>**Citrinin** (yellow) | | I | No | [4] |
| *P. cyclopium* | **Viomellein** (reddish-brown)<br>**Xanthomegnin** (orange) | Penicillic acid | unknown | No | [10] |
| *P. discolor* | Uncharacterized | Chaetoglobosin A, B & C | unknown | No | [10] |
| *P. echinulatum* | Uncharacterized (yellow) | Territrems | unknown | No | [10] |
| *P. flavigenum* | Xanthocillins | unknown | unknown | TBI | [10] |
| *P. freii* | **Viomellein** (reddish-brown)<br>**Vioxanthin**<br>**Xanthomegnin** (orange) | | unknown | No | [10] |
| *P. herquei* | Atrovenetin (yellow)<br>Herqueinones (red and yellow) | unknown | unknown | Yes | [13] |
| *P.oxalicum* | Arpink red™-anthraquinone derivative (red)<br>**Secalonic acid D** (yellow) | | unknown | No | [3,14] |
| *P. paneum* | Uncharacterized (red) | Botryodiploidin<br>Patulin<br>Roquefortine C | unknown | No | [10] |
| *P. persicinum* | Uncharacterized (Cherry red) | Roquefortine C | unknown | No | [10] |
| *P. viridicatum* | **Viomellein** (reddish-brown)<br>**Vioxanthin**<br>**Xanthomegnin** (orange) | Penicillic acid<br>Viridic acid | unknown | No | [10] |
| ***Talaromyces* (anamorph *Penicillium* subgenus *Biverticillium*)** | | | | | |
| *T. macrosporus* | Mitorubrin (yellow) | Duclauxin<br>Islanditoxin | unknown | No | [15] |
| *P. aculeatum* | Uncharacterized | | unknown | Yes | [un- pub-lished] |
| *P. crateriforme* | Uncharacterized | Rubratoxin<br>Rugulovasine A &B<br>Spiculisporic acid | unknown | No | [16] |
| *P. funiculosum* | Uncharacterized | | unknown | Yes | [17] |
| *P. islandicum* | **Emodin** (yellow)<br>**Erythroskyrin** (orange-red)<br>**Luteoskyrin** (yellow)<br>**Skyrin** (orange) | Cyclochlorotine<br>Islanditoxin<br>Rugulosine<br>Rugulovasine A & B | unknown | No | [16] |

**Table 1: Comprehensive list of polyketide pigment producing *Penicillium* and *Aspergillus* species and their known teleomorphs.**

| | | | | | |
|---|---|---|---|---|---|
| *P. marneffei* | Mitorubrinol<br>Monascorubramine (purple-red)<br>Purpactin<br>Rubropunctatin (orange)<br>**Secalonic acid D** (yellow) | | 3 | No | [un- published] |
| *P. pinophilum* | Uncharacterized | | unknown | Yes | [17] |
| *P. purpurogenum* | Mitorubrin (yellow)<br>Mitorubrinol (orange-red)<br>PP-R (purple red)<br>Purpurogenone (yellow-orange) | | 1 | Yes | [5,16,18] |
| *P. rugulosum* | **Rugulosin** (yellow) | | 1 | No | [9,16] |
| *P. variabile* | **Rugulosin** (yellow) | | unknown | No | [9,16] |
| ***Eurotium* (anamorph *Aspergillus* subgenus *Aspergillus*)** | | | | | |
| *E. amstelodami* | Auroglaucin (orange)<br>Erythroglaucin (red)<br>Flavoglaucin (yellow)<br>**Physcion** (yellow) | Echinulin | 1 | No | [9] |
| *E. chevalieri* | Auroglaucin<br>Erythroglaucin<br>Flavoglaucin<br>**Physcion** (yellow) | Echinulin | 1 | No | [9] |
| *E. herbariorum* | Aspergin (yellow)<br>Flavoglaucin (yellow)<br>**Physcion** (yellow) | Echinulin | 1 | No | [9] |
| ***Aspergillus* section *Circumdati*** | | | | | |
| *A. ochraceus* | **Viomellein** (reddish-brown)<br>**Vioxanthin**<br>**Xanthomegnin** (orange) | Ochratoxin A<br>Penicillic acid | 1 | No | [19] |
| *A. melleus* | **Rubrosulphin** (red)<br>**Viomellein** (reddish-brown)<br>**Viopurpurin** (purple)<br>**Xanthomegnin** (orange) | | unknown | No | [19] |
| *A. sulphureus* | **Rubrosulphin** (red)<br>**Viomellein** (reddish-brown)<br>**Viopurpurin** (purple)<br>**Xanthomegnin** (orange) | | unknown | No | [19] |
| *A. westerdijkiae* | **Rubrosulphin** (red)<br>**Viomellein** (reddish-brown)<br>**Viopurpurin** (purple)<br>**Xanthomegnin** (orange) | Ochratoxin A<br>Penicillic acid | unknown | No | [19] |
| ***Aspergillus* section *Nigri*** | | | | | |
| *A. niger* | Flavioline (orange-red),<br>Nnaptho-γ-pyrones (yellow) | Fumonisins<br>Ochratoxin A | 1 | No | [20] |
| *A. sclerotioniger* | Uncharacterized yellow | Ochratoxin A | unknown | No | [20] |
| ***Emericella* (anamorph *Aspergillus* subgenus *Nidulantes*, section *Nidulantes* and section *Versicolores*)** | | | | | |
| *Em. falconensis* | Falconensins C-H (yellow)<br>Falconensones (Yellow)<br>Zeorin (yellow) | unknown | unknown | TBI | [21] |
| *Em. purpurea* | Epurpurins A-C (yellow) | unknown | unknown | TBI | [22] |
| *A. versicolor* | **Sterigmatocystin** (yellow) | | 1 | No | [23] |

[1] BSL-1: saprobes or plant pathogens occupying non-vertebrate ecological niches, or commensals. Infections are coincidental, superficial, and non-invasive or mild. BSL-3: pathogens potentially able to cause severe, deep mycoses in otherwise healthy patients.
[2] Keys to selection; Yes: preselected as a possible source of pigments, No: not selected as a possible source of pigments, TBI: to be investigated as a possible source of pigments.

The *a priori* species-specific major pigment and/or toxic metabolite profiles and the BioSafety Level (BSL) classification of the fungal species are highlighted and form the basis for selection/de-selection of the species as a potential source of pigment production.

**Table 2: Selected ascomycetous fungi and their species-specific polyketide pigment and/or toxic metabolite profiles.**

| Fungal species | Pigment composition (colour). Toxic colored compounds in bold | Major known mycotoxic uncolored metabolites | BioSafety Level (BSL) classification[1] [32] | Comment[2] | Ref. |
|---|---|---|---|---|---|
| *Fusarium acuminatum* | Antibiotic Y (yellow)<br>Aurofusarin (red) | Enniatins<br>Moniliformin | unknown | No | [24] |
| *F. avenaceum* | Antibiotic Y (yellow)<br>Aurofusarin (red) | Enniatins<br>Moniliformin<br>2-amino-14,16-di-methyloctadecan-3-ol | unknown | No | [24] |
| *F. culmorum* | Aurofusarin (red)<br>Fuscofusarin (yellow)<br>Rubrofusarin (red) | Butenolide<br>Fusarin C<br>Trichothecenes<br>Zearalenone | unknown | No | [24] |
| *F. fujikuroi* | Bikaverin (red)<br>Norbikaverin (red)<br>O-demethylanhydrofusarubin (red) | Fumonisins<br>Fusaric acid<br>Gibberellins<br>Moniliformin | I | No | [25] |
| *F. graminearum* | Aurofusarin (red)<br>Rubrofusarin (red) | Butenolide<br>Fusarin C<br>Trichothecenes<br>Zearalenone | unknown | No | [24] |
| *F. oxysporum* | 2,7-dimehoxy-6-(acetoxyethyl)juglone (yellow)<br>Bikaverin (red)<br>Bostrycoidin (red)<br>Nectriafurone (yellow)<br>Norjavanicin (red)<br>O-methyl-6-hydroxynorjavanicin (yellow)<br>O-methylanhydrofusarubin (orange-red)<br>O-methylfusarubin (red)<br>O-methyljavanicin | Fumonisins<br>Fusaric acid<br>Moniliformin | 2 | No | [25] |
| *F. poae* | Aurofusarin (red) | Enniatins<br>Fusarin C<br>Trichothecenes | unknown | No | [24] |
| *F. sambucinum* | Aurofusarin (red) | Trichothecenes | unknown | No | [24] |
| *F. solani* | Fusarubin (red)<br>Isomarticins (red)<br>O-ethylfusarubin (red)<br>O-methyldihydrofusarubin (red) | | 2 | No | [25] |
| *F. sporotrichioides* | Aurofusarin (red)<br>Lycopene | Enniatins<br>Trichothecenes | unknown | No | [24] |
| *F. stilboides* | Antibiotic Y (yellow)<br>Aurofusarin (red)<br>Nectriafurone (yellow) | Enniatins | unknown | No | [un-published] |
| *F. tricinctum* | Antibiotic Y (yellow)<br>Aurofusarin (red) | Fusarin C<br>Moniliformin | unknown | No | [24] |
| *F. venenatum* | Aurofusarin (red)<br>Rubrofusarin (red) | Trichothecenes | unknown | No | [24] |
| *F. verticillioides* | Fusarubin<br>O-demethylfusarubin<br>O-methyljavanicin<br>O-methylsolaniol (orange-red) | Fumonisins<br>Fusaric acid<br>Moniliformin | unknown | No | [25] |
| *Alternaria dauci* | Uncharacterized (red) | unknown | I | TBI | [26] |
| *Alt. porri* | Altersolanol A (yellow-orange) | unknown | I | TBI | [26] |

**Table 2: Selected ascomycetous fungi and their species-specific polyketide pigment and/or toxic metabolite profiles.** *(Continued)*

| | | | | | |
|---|---|---|---|---|---|
| *Alt. solani* | Altersolanol A (yellow-orange) | unknown | I | TBI | [26] |
| *Alt. tomatophila* | Altersolanol A (yellow-orange) | unknown | unknown | TBI | [26] |
| *Cladosporium cladosporioides* | Calphostins A, B, C, D, I (red) | unknown | I | TBI | [29] |
| *Cordyceps unilateralis* | 3,5 8-TMON* (red)<br>4-O-methyl erythrostominone (red)<br>Deoxyerythrostominol (red)<br>Deoxyerythrostominone (red)<br>Epierythrostominol (red)<br>Erythrostominone (red) | unknown | unknown | TBI | [30] |
| *Curvularia lunata* | Chrysophanol (red)<br>Cynodontin (bronze)<br>Helminthosporin (maroon) | unknown | I | TBI | [4] |
| *Drechslera* spp. | Catenarin (red)<br>Cynodontin (bronze)<br>Helminthosporin (maroon)<br>Tritisporin (redish-brown) | unknown | unknown | TBI | [4] |
| *Epicoccum nigrum* | Carotenoids<br>Chromanone (yellow)<br>Epicoccarines A & B<br>Epicocconone (fluorescent yellow)<br>Epipyridone (red)<br>Flavipin (brown)<br>Isobenzofuran derivatives (yellow to brown)<br>Orevactaene (yellow) | unknown | unknown | Yes | [5,27,28] |
| *Paecilomyces sinclairii* | Uncharacterized (red) | unknown | unknown | TBI | [31] |
| **Polyketide pigment producer of Asia** | | | | | |
| *Monascus pilosus* | **Citrinin** (yellow) | | I | Banned in the EU & the US | [6] |
| *M. purpureus* | Ankaflavin (yellow)<br>**Citrinin** (yellow)<br>Monascin<br>Monascorubramine<br>Monascorubrin<br>Rubropunctamine (purple-red)<br>Rubropunctatin (orange) | Monascopyridine A & B | I | Banned in the EU & the US | [6] |
| *M. ruber* | Ankaflavin (yellow)<br>**Citrinin** (yellow)<br>Monascin<br>Monascorubramine<br>Monascorubrin<br>Rubropunctamine (purple-red)<br>Rubropunctatin (orange) | | I | Banned in the EU & the US | [6] |

[1] BSL-1: saprobes or plant pathogens occupying non-vertebrate ecological niches, or commensals. Infections are coincidental, superficial, and non-invasive or mild. BSL-2: Species principally occupying non-vertebrate ecological niches, but with a relatively pronounced ability to survive in vertebrate tissue. In severely immuno-compromised patients they may cause deep opportunistic mycoses. Also pathogens causing superficial infections are in this category.

[2] Keys to selection; Yes: preselected as a possible source of pigments, No: not selected as a possible source of pigments, TBI: to be investigated as a possible source of pigments.

The *a priori* major metabolite profiles and the BioSafety Level (BSL) classification of the fungal species are highlighted and form the basis for selection/de-selection of the species as a potential source of pigment production.

**Table 3: Potentially safe and toxin-free cell factories belonging to *Penicillium* subgenus *Biverticillium* that produce *Monascus*-like pigments.**

| Fungal species Serial No. | IBT Culture Collection # | Other culture collection # | Source of isolation | Pigments identified | Known mycotoxins |
|---|---|---|---|---|---|
| *Penicillium purpurogenum* | | | | | |
| **1**. | 11180[1,2] | - | Unknown | Monascorubramine[3], PP-R[1,3] | None |
| **2**. | 11181[2] | CBS 123796 | Pepper fruit | N-glutarylrubropunctamine[4]<br>N-glutarylmonascorubramine[4] | None |
| **3**. | 21347 | - | Human saliva | PP-R[5] | None |
| **4**. | 23082 | RMF 81.01 | Soil | PP-R[3] | None |
| **5**. | 3645[2] | IMI 90178 | Unknown | N-glutarylmonascorubramine[6] | None |
| **6**. | 3967[2] | NRRL 1147 | Unknown | Monascorubramine[7]<br>Monascin | None |
| *Penicillium aculeatum* | | | | | |
| **7**. | 14259 | NRRL 2129 | Weathering fabric | Monascorubrin[5] | None |
| **8**. | 14263[1] | FRR 1802 | Soil | Monascorubrin[1,3]<br>Xanthomonasin A[1,3]<br>Threonine derivative of rubropunctatin[1,5] | None |
| *Penicillium funiculosum* | | | | | |
| **9**. | 3954 | NRRL 2119 | Unknown | Ankaflavin[8] | None |
| *Penicillium pinophilum* | | | | | |
| **10**. | 13104[1] | ATCC 9644 | Radio set | Monascorubrin[1,3] | None |

[1]Previously reported [5,33]; [2] Tested for the pigment producing ability in the liquid media and was positive; [3]On YES Agar; [4] In M1 liquid medium; [5] On CYA agar; [6] In M2 liquid medium; [7] On OAT agar; [8] On MEA agar.

Also, genomic approaches of selection of potential polyketide pigment producers may not be useful at this point of time when none of the fungal polyketide pigment producers are fully genome sequenced yet. Moreover, the problem of annotating correct gene sequences is not to be overlooked especially due to the variation in the domain of polyketide synthases; two or more polyketide synthases are involved in the biosynthesis of such complex secondary metabolites as pigments. Chemotaxonomy uses secondary metabolite profiles of filamentous fungi as the secondary metabolites have a differentiation capability at a genus and species level [36]. This has been used successfully and resulted in a lot of *a priori* knowledge about filamentous fungi – their bio- and chemical diversity, ecological niche, the species-specific metabolite profiles, and optimal media and growth conditions for secondary metabolites (including pigments) production [37]. Phylogeny as a taxonomic tool, whereby the partial sequences of the household genes such as β-tubulin are used, on the other hand, cannot predict the functional trait of the organisms as described by Samson et al. [38]. Therefore, chemotaxonomic selection would form an essential element of high throughput screening programmes as the use of *a priori* knowledge of species-specific metabolite/pigment and/or mycotoxin profiles ensures a quick and efficient way of selecting potentially safe pigment producers from a vast bio- and chemodiversity of filamentous fungi. The purpose of the present work was to demonstrate the chemotaxonomic selection and de-selection approach in the pigment producing fungi whereby specific mycotoxin production was shown in the producer or the non-producer in addition to the *a priori* knowledge of their mycotoxin and pigment profile. Thus, the starting point of our selection approach was the use of chemotaxonomic knowledge about the polyketide pigment producing fungi (Table 1 and Table 2). We used this as a key to show that only a handful of pigment producers are worth exploring as potentially safe fungal cell factories for polyketide pigment production (Table 3). The LC-DAD-MS was successfully used as a powerful tool to check the presence or the absence of mycotoxins using authentic standard in the run and to identify pigments based on UV-vis and mass spectra. Since, pigments have characteristic UV-vis spectra, the use of UV-vis spectra in the identification and de-replication was shown to be quite handy. The two examples (Figure 2, Figure 3 and Figure 4), not only demonstrated our chemotaxonomic selection/de-selection approach but also proved the use of LC-DAD-MS as an efficient chemotaxonomic tool. To the best of our knowledge, this unique and rational approach is not yet demonstrated as a selection criterion for fungal cell factories. Thus, it signifies our findings to emphasize on the appropriate use of chemotaxonomy in the discovery of novel and potentially safe cell factories for the industrially useful polyketides including pigments for food use. However, the toxicological studies are still imperative for the

**Figure 1**
***Penicillium purpurogenum* IBT 11180 on YES agar after 7 days of incubation showing extracellular pigment production**.

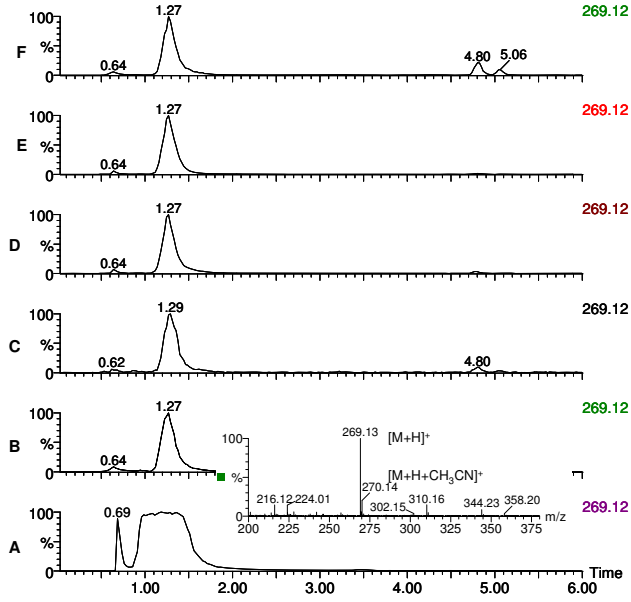



**Figure 2**
**Chromatograms of rugulovasine A and B and pigment extracts of *Penicillium crateriforme***. The extracted ion chromatograms (m/z 269.12) of standard rugulovasine A and B and pigment extracts of *P.crateriforme* IBT 5015 grown on 5 different solid agar media depict the presence of rugulovasine A and B with its mass spectrum. 2A, standard rugulovasine A and B; 2B, pigment extract from CYA; 2C, pigment extract from MEA; 2D, pigment extract from PD; 2E, pigment extract from OAT; 2F, pigment extract from YES.

final approval of the product and the process that would entail the use of these pre-selected potentially safe cell factories especially for the food use.

### *New potentially safe fungal cell factories for the production of Monascus-like pigments*

Several of the already known *Monascus*-like pigments (Table 3, Figure 4 and Figure 5) were identified in *Penicillium* species that do not produce any known mycotoxins when grown under the laboratory conditions used in this study. It could be noted that these Penicillia are closely related according to Raper and Thom [39], and Pitt [40] and that they are also closely related according to phylogenetic principles [41] but distantly related to *Monascus* species.

The production of extracellular pigments by some of the tested cell factories in the liquid media using a solid support has added to the value of these cell factories as future production strains. N-glutarylmonascorubramine and N-glutarylrubropunctamine have been reported as water-soluble extracellular pigments of *Monascus ruber* [42-44]. The identification of N-glutarylmonascorubramine was based on mass and UV-vis spectra that matched quite well with the previous reports [42,44]. The mass spectra of N-glutarylrubropunctamine matched very well with the one reported by Hajjaj et al. [44]. The UV-vis spectrum of N-glutarylrubropunctamine was very similar to that of N-glutarylmonascorubramine as the two compounds differed only in their aliphatic side chain. Multiple extracellular pigments (Figure 6 and Figure 7) were formed from a complex nitrogen source such as yeast extract (as in case of N1 medium) or a combination of such complex nitrogen sources as corn steep liquor and yeast extract (as in case of N2 medium) and rubropunctatin and/or monascorubrin in the media possibly by Schiff base formation type of reaction mechanism. This mechanism was reported previously [42,44] for the formation of these water soluble pigments from their counter parts which are aminophilic in nature being azaphilones. Hajjaj et al. [44] reported the formation of 4 pigment molecules that constituted 91% of the pigment production when only one nitrogen source (monosodium glutamate) in the medium was used with 20 g/L glucose. Our results indicate formation of much more than 4 pigment components for both of the tested Penicillia (Figure 6 and Figure 7) possibly due to the availability of several amino acids from the complex nitrogen source/s provided in the media. Thus, in the future it is possible to obtain desired number of pigment components by using defined nitrogen sources that would provide specific amino acid/s moiety to be incor-

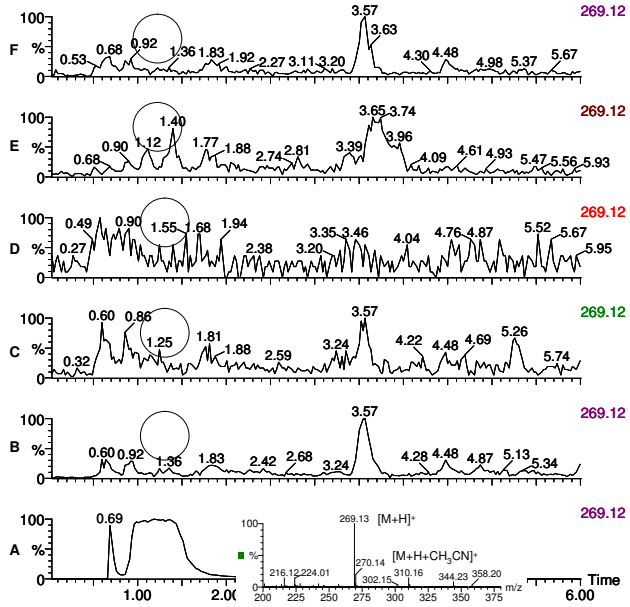



**Figure 3**
**Chromatograms of rugulovasine A and B and pigment extracts of *Penicillium aculeatum*.** The extracted ion chromatograms (m/z 269.12) of standard rugulovasine A and B and pigment extracts of *P. aculeatum* IBT 14259 grown on 5 different solid agar media depict the absence of rugulovasine A and B with its mass spectrum. 3A, standard rugulovasine A and B; 3B, pigment extract from YES; 3C, pigment extract from PD; 3D, pigment extract from OAT; 3E, pigment extract from MEA; 3F, pigment extract from CYA.

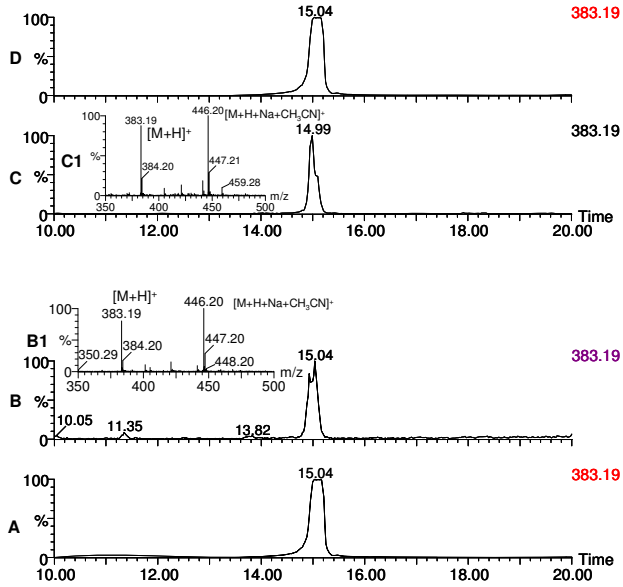



**Figure 4**
**Chromatograms of monascorubrin and pigment extracts of *Penicillium aculeatum* and *Penicillium crateriforme*.** The extracted ion chromatogram (m/z 383.19) of standard monascorubrin and pigment extracts of *P. aculeatum* IBT 14259 and *P. crateriforme* IBT 5015 on CYA depict the presence of monascorubrin with its mass spectrum. Bottom panel A, standard monascorubrin; B, pigment extract of *P. crateriforme* IBT 5015 on CYA; B1, mass spectrum of monascorubrin. Top panel C, pigment extract of *P. aculeatum* IBT 14259 on CYA; C1, mass spectrum of monascorubrin; D, standard monascorubrin.

porated into the azaphilone pigments to form water soluble extracellular pigments. As these water soluble extracellular pigments are more stable than their orange counterparts [45], this is very significant for the biotechnological production of such natural colorants in these newly discovered cell factories Thus it can be inferred that the discovery of such potentially safe cell factories for polyketide natural food colorants using chemotaxonomic approach is a milestone that would redefine the biotechnological production of food colorants derived from such an agro-independent source of colorants as filamentous fungi. Moreover, this also can tackle the current issue of restricted use of *Monascus* pigments due to the co-production of toxic metabolites.

## Conclusion

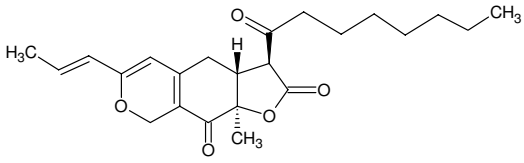
In conclusion, we have shown that the use of chemotaxonomic tools and *a priori* knowledge of fungal extrolites is a rational approach towards selection of potentially safe polyketide natural colorant producing fungal cell factories considering the enormous chemical diversity and biodiversity of ascomycetous fungi. This approach could be very handy for the selection of potentially safe fungal cell factories not only for polyketide pigments but also for the other industrially important polyketides; the molecular and genetic basis for the biosynthesis of which has not yet been examined in detail. In addition, 4 out of the 10 chemotaxonomically selected promising *Penicillium* strains were shown to produce extracellular pigments in the liquid media using a solid support; two of the pigments were identified as *Monascus* pigment derivatives N-glutarylrubropunctamine and N-glutarylmonascorubramine, by means of LC-DAD-MS chromatography. Work is underway for the evaluation of a few of these promising cell factories for the controlled and robust production of such polyketide natural food colorants.

## Methods

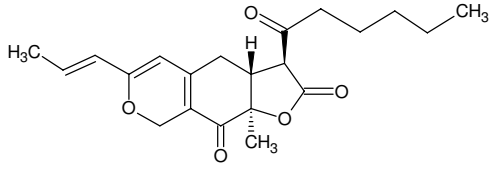
### *Pre-selection of fungi, media, and cultivation conditions*

All fungal isolates used in this study were procured from the IBT Culture Collection at Center for Microbial Biotechnology, Technical University of Denmark, Kgs. Lyn-

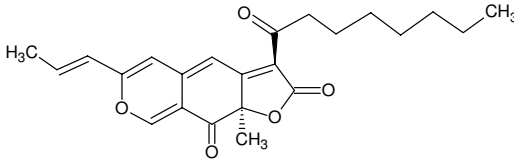
Ankaflavin ($C_{23}H_{30}O_5$, 386.48)



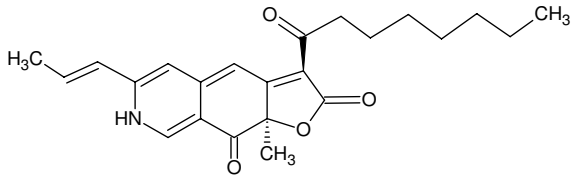
Monascin ($C_{21}H_{26}O_5$, 358.43)



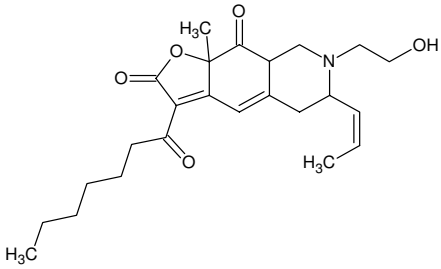
Monascorubrin ($C_{23}H_{26}O_5$, 382.45)



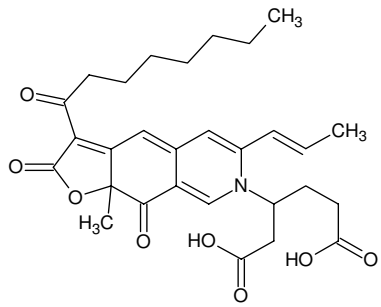
Monascorubramine ($C_{23}H_{27}NO_4$, 381.46)



PP-R; 7-(2-Hydroxyethyl)-monascorubramine ($C_{25}H_{31}NO_5$, 426.23)



N-glutarylmonascorubramine ($C_{28}H_{33}NO_8$, 511.23)



N-glutarylrubropunctamine ($C_{26}H_{29}NO_8$, 483.20)

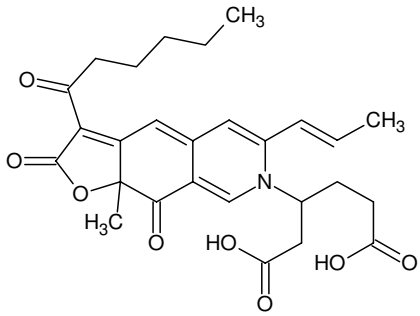


**Figure 5**
**Structures of the pigments detected and identified in the present study**. Formula and calculated nominal masses are shown in the parentheses.

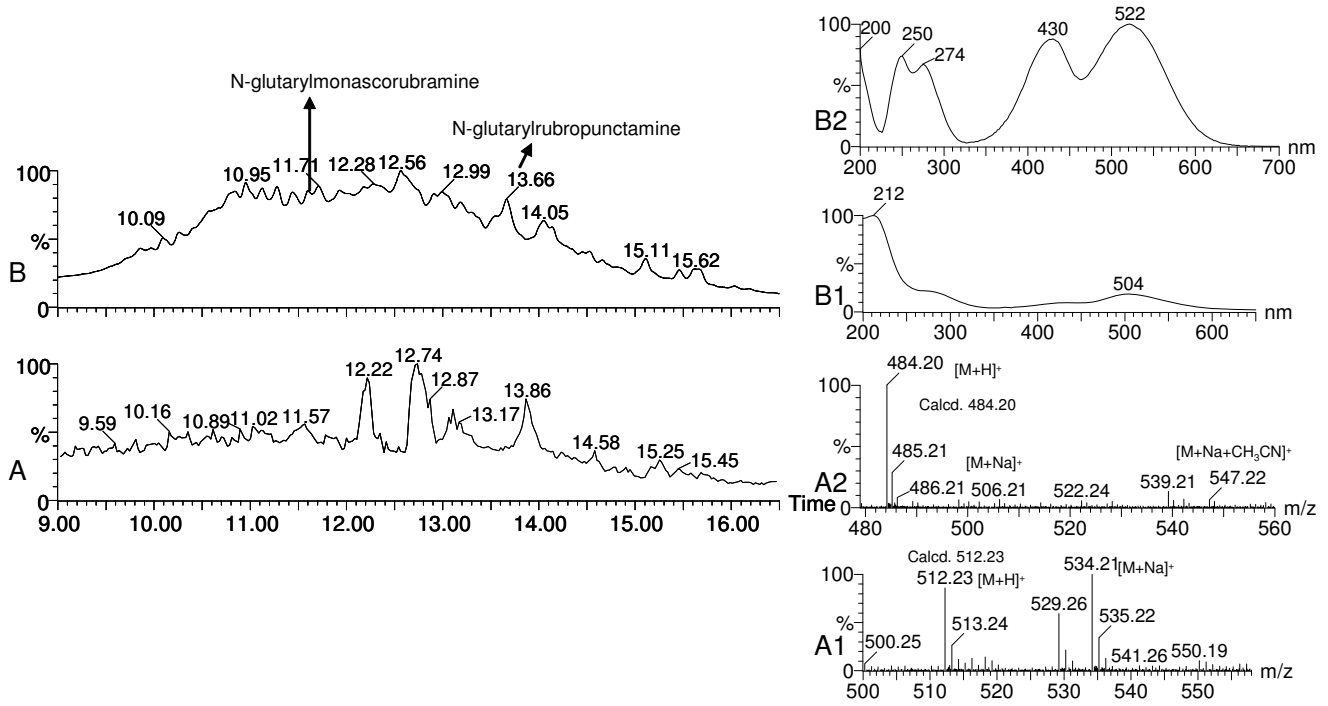



**Figure 6**

**Presence of N-glutarylmonascorubramine and N-glutarylrubropunctamine in the extracellular pigment extract of *Penicillium purpurogenum* IBT 11181**. The strain was grown in N1 liquid medium with LECA as a solid support. Total ion chromatogram (m/z 100–900), on X-axis time is shown, and on Y-axis % of the ion count (A), and UV-vis chromatogram at 390–700 nm, on X-axis time is shown, and on Y-axis % of the sum of the absorbencies, a relative value (B), mass spectrum (A1) and UV-vis spectrum of N-glutarylmonascorubramine (B1), mass spectrum (A2) and UV-vis spectrum of N-glutarylrubropunctamine (B2).

gby, Denmark. The fungal isolates were listed by the IBT numbers. *Penicillium aculeatum* IBT 14259 and *P. crateriforme* IBT 5015 was cultivated on five different solid media *viz*; Yeast extract sucrose (YES) agar; Malt extract agar (MEA), Oatmeal (OAT) agar, Potato dextrose (PD) agar and Czapek-Dox yeast autolysate (CYA) agar [46]. The cultures were incubated in the dark at 25°C for 7 days.

For the liquid media with the solid support, CZ medium [46] with 0.5% yeast extract (designated as N1) was used in case of *Penicillium purpurogenum* IBT 11181. N2 medium; where the basal medium was kept the same as in CZ medium except that the carbon sources and nitrogen sources were (g/L): potato starch, 2.75; lactose, 5.5; ammonium nitrate, 1.55; corn steep liquor, 1.55, was used for *Penicillium purpurogenum* IBT 3645. The initial pH of the medium was adjusted to 5.5 using 0.1 M HCl. Approximately 8–9 grams of Light Expanded Clay Aggregates (LECA) were used as solid support retained within a tea paper filter (Schur Inventure A/S, Vejle, Denmark, extra fine pores capacity 16.5 g/m$^2$, locally purchased) and sterilized by autoclaving. Spores harvested from a week old culture plate (CYA) with a concentration (3 × 10$^5$/ml) were inoculated directly onto the LECA contained within the tea filter, which was then transferred into 300 ml baffled Erlenmeyer culture flasks comprising 100 mL of the N1 or N2 media. The experiment was performed in duplicate. The cultures were incubated at 25°C in the dark under shaking conditions (120 rpm).

### *Extraction of fungal pigments*

In case of solid media, extraction was carried out by a modified version of the micro-scale extraction method [47], where 6 mm plugs were extracted ultrasonically with solvent containing ethyl acetate, dichloromethane, and methanol in a ratio of 3:2:1 (v/v/v) with 1% formic acid. The extract was evaporated to dryness in a rotational vacuum concentrator (RVC; Christ Martin, Osterode, Germany). Residue was re-dissolved in 400 μL methanol, in an ultrasonic bath (Branson 2510, Kell-Strom, Wethers-

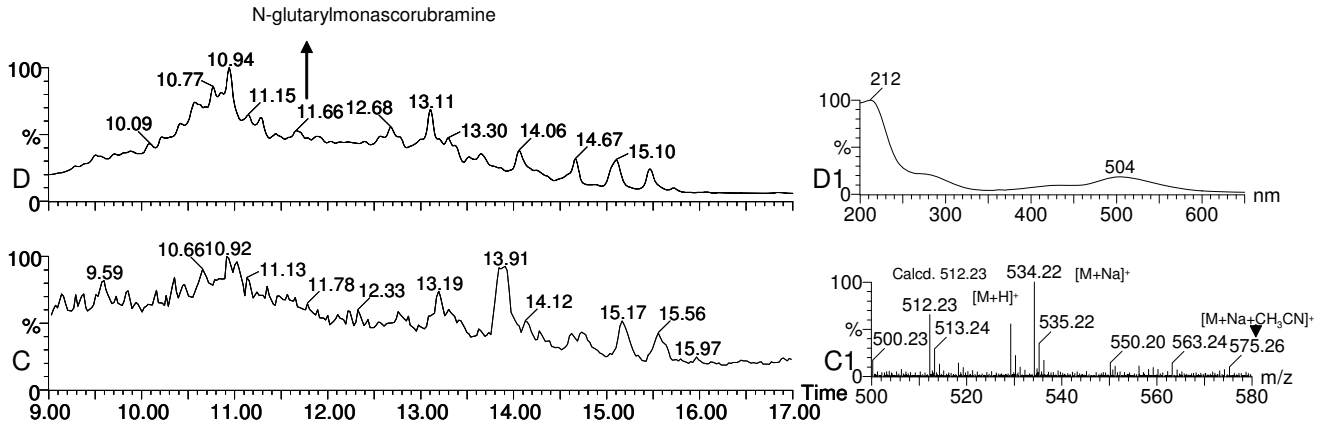



**Figure 7**
**Presence of N-glutarylmonascorubramine in the extracellular pigment extract of *Penicillium purpurogenum* IBT 3645**. The strain was grown in N2 liquid medium with LECA as a solid support. Total ion chromatogram (m/z 100–900), on X-axis time is shown, and on Y-axis % of the ion count (C), and UV-vis chromatogram at 390–700 nm, on X-axis time is shown, and on Y-axis % of the sum of the absorbencies, a relative value (D), mass spectrum (C1) and UV-vis spectrum of N-glutarylmonascorubramine (D1).

field, USA) for 10 minutes, and filtered through a 0.45 μm PTFE syringe filter (SRI, Eatontown, New Jersey, USA). This extract was used for chromatographic analysis.

In case of liquid media with solid support, after 7 days of incubation the tea filter comprising the majority of the fungal biomass adhered to LECA was removed from the flask and the fermentation broth containing extracellular pigments was filtered through Whatman filter paper # 1 to remove the residual biomass.

### *Evaluation of pigment composition from the fermentation broth*

The filtrate was subject to clean-up by solid phase extraction Strata-X-C 33 μm cation mixed mode polymer columns (60 mg 1 mL, Phenomenex, Torrence, California, USA). 1.2 mL of methanol was used for conditioning followed by 1.2 mL of distilled water for calibration. 1.2 mL of samples, acidified with 0.1% phosphoric acid (1: 6 v/v) were loaded in a vacuum manifold, and washed with 0.1% phosphoric acid. Elution of the pigment mixture, bound in the matrix of the cartridges, was carried out using methanol which could elute neutral and acidic components. The pigment extract so obtained was subjected to high resolution LC-DAD-MS analysis.

### *Chromatographic analysis*

High-resolution LC-DAD-MS was performed on an Agilent HP 1100 liquid chromatograph (LC) system with a photodiode array detector (DAD) and a 50 × 2 mm i.d., 3 μm, Luna $C_{18}$ II column (Phenomenex, Torrance, CA). The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, U.K.) with a Z-spray electrospray ionization (ESI) source, and a LockSpray probe and controlled by the MassLynx 4.0 software. MS system was operated in the positive ESI mode using a water-acetonitrile gradient system starting from 15% acetonitrile, which was increased linearly to 100% in 20 minutes with a holding time of 5 minutes. The water was buffered with 10 mM ammonium formate and 20 mM formic acid and the acetonitrile with 20 mM formic acid. The instrument was tuned to a resolution > 7000 (at half peak height). The method is well established at our research center for the metabolite profiling of filamentous fungi grown on solid media. It is described by Nielsen et al. [48].

For the extracellular pigments extracted from the liquid media, the solvent system used was water with 0.1% formic acid and acetonitrile with 0.1% formic acid. The gradient started from 5% acetonitrile and increased to 100% in 20 minutes and was hold at 100% for 2 minutes. The MS conditions were the same as mentioned earlier.

### *Analysis of LC-DAD-MS Data*

The coloured components in the pigment extract were detected in the UV-vis chromatogram of 390–700 nm. The identification of N-glutarylmonascorubramine and N-glutarylrubropunctamine was based on both UV-vis and mass spectra from total ion chromatogram (m/z 100–900) from positive ion electro spray. The UV-vis spectrum was obtained after background subtraction. The DAD-MS

data for N-glutarylmonascorubramine and N-glutarylrubropunctamine is shown below:

N-glutarylmonascorubramine was detected as *m/z* 512.23 $[M + H]^+$and confirmed by the adducts *m/z* 534.22 $[M + Na]^+$ and *m/z* 575.26 $[M + Na + CH_3CN]^+$. The UV-vis spectrum was $\lambda_{max}$: 212, with a shoulder at 272, 420 and 504.

N-glutarylrubropunctamine was detected as *m/z* 484.20 $[M + H]^+$and confirmed by the adducts *m/z* 506.21 $[M + Na]^+$ and *m/z* 547.22 $[M + Na + CH_3CN]^+$. The UV-vis spectrum was $\lambda_{max}$: 200, 250, 274, 430 and 522.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

SASM performed the experiments and drafted the manuscript, ASM helped in the writing of the manuscript. UT and JCF contributed to the chemotaxonomic selection/deselection part in Table 1. JCF contributed with his chemotaxonomic expertise in *Penicillium* subgenus *Biverticillium*. All authors read and approved the final manuscript.

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# APPENDIX B

Supplementary information for Chapter 3.

**List of colored mycotoxins and secondary metabolites of ascomycetous fungi**.
(Compiled from Nielsen and Smedsgaard, 2003, J. Chromat. A, 1002: 111-136)

| Metabolite | Formula | Retention time (min) | UV-Vis absorption (nm) in % of UV-max | Monoisotopic mass |
|---|---|---|---|---|
| *Aflatoxins and their precursors* | | | | |
| Averufin | $C_{20}H_{16}O_7$ | 25.65 | 224(95), 292(100), 268(55), 254sh, 322(24), 456(32) | 368.0896 |
| Norsolorinic acid | $C_{20}H_{18}O_7$ | 31.08 | 236(100), 270sh, 282sh, 308(91), 468(43) | 370.1053 |
| *Others* | | | | |
| Alizarin | $C_{14}H_8O_4$ | 14.60 | 228shh, 248(100), 280(68), 328(14), 432(26) | 240.0423 |
| Altersolanol A | $C_{16}H_{16}O_8$ | 2.90 | 220(100), 268(41), 430(12) | 336.0845 |
| Amphotericin B | $C_{47}H_{73}NO_{17}$ | 21.39 | 228(10), 340(25), 364(52), 384(92), 408(100) | 923.4879 |
| Anhydrofusarubin | $C_{15}H_{12}O_6$ | 21.45 | End(100), 236(88), 288(87), 348sh, 540(47) | 288.0634 |
| Ascochitine | $C_{15}H_{16}O_5$ | 18.75 | 220(81), 262sh, 280(100), 326(15), 242(15), 416(38) | 276.0998 |
| Asperthecin | $C_{15}H_{10}O_8$ | 10.00 | End(75), 236(88), 260(100), 286(55), 316(36), 484(56), 506sh | 318.0376 |
| Bostrycin | $C_{16}H_{16}O_8$ | 3.95 | 228(100), 304(30), 482sh, 540(26), 536sh | 336.0845 |
| Bostrycoidin | $C_{15}H_{11}NO_5$ | 16.90 | 204(96), 252(100), 324(21), 472sh, 492(29), 526sh | 285.0637 |
| Brevianamide A | $C_{21}H_{23}N_3O_3$ | 10.29 | 232broad(100), 262sh, 408(13) | 365.1739 |
| Calphostin C | $C_{44}H_{38}O_{14}$ | 24.66 | 224(100), 268(50), 476(41), 543(20), 584(20) | 790.2262 |
| Carlosic acid | $C_{10}H_{12}O_6$ | 1.26 | 232(100), 264(94), 316sh, 408(8) | 228.0634 |
| Catenarin | $C_{15}H_{10}O_6$ | 22.70 | 232(100), 256(50), 276(52), 302(32), 464sh, 492(42), 520sh | 286.0477 |
| Chrysazin | $C_{14}H_8O_4$ | 20.84 | 224(100), 252(88), 284sh, 428broad(48) | 240.0423 |
| Chrysophanol | $C_{15}H_{10}O_4$ | 23.98 | 224(100), 256(65), 280sh, 288sh, 432broad(30) | 254.0579 |
| Citreomontanin | $C_{23}H_{28}O_3$ | 33.35 | 232(40), 268(39), 330(35), 412(100) | 352.2038 |
| Cladofulvin | $C_{30}H_{18}O_{10}$ | 22.60 | End(80), 232(95), 268(100), 294sh, 448(44) | 538.0900 |
| Cynodontin | $C_{15}H_{10}O_6$ | 28.04 | 236(100), 284sh, 296(18), 488sh, 516(40), 544(38), 552(39) | 286.2408 |
| Deoxybostrycin | $C_{16}H_{16}O_7$ | 8.9 | 228(100), 304(28), 474sh, 500(30), 536(18) | 320.0896 |
| Dermoglaucin | $C_{16}H_{12}O_6$ | 18.60 | 212(100), 284(99), 432(40) | 300.0634 |
| Dês-methoxyviridiol | $C_{19}H_{16}O_5$ | 9.17 | 232(40), 280(100), 320(12), 332(11), 402(18) | 324.0998 |
| Emodin | $C_{15}H_{10}O_5$ | 20.14 | 222(100), 258sh, 268(55), 288(64), 440(38) | 270.0528 |
| Erythroglaucin | $C_{16}H_{12}O_6$ | 27.66 | 230(100), 256(52), 276(53), 304(41), 370sh, 492(42), 518sh, | 300.0634 |
| Fusarubin | $C_{15}H_{14}O_7$ | 11.71 | 228(100), 304(30), 474sh, 500(28), 526sh | 306.0740 |
| Helminthosporin | $C_{15}H_{10}O_5$ | 26.14 | 231(100), 255(49), 288(23), 484(30), 518sh | 270.0528 |
| Ω-hydroxypachybasin | $C_{15}H_{10}O_4$ | 14.87 | End(85), 222(61), 258(100), 277sh(45), 334(10), 440(20) | 254.0579 |

| | | | | |
|---|---|---|---|---|
| Iodinine | $C_{12}H_8N_2O_4$ | 14.64 | 288(100), 344(8), 524(8) | 244.0484 |
| Islandicin | $C_{15}H_{10}O_5$ | 27.88 | 230(100), 252(69), 292(23), 264sh, 492(35) | 270.0528 |
| Isoemodin | $C_{15}H_{10}O_5$ | 15.12 | End(55), 224(100), 256(64), 286sh, 432(30) | 270.0528 |
| Isomarticin | $C_{18}H_{16}O_9$ | 12.33 | 228(100), 304(30), 472sh, 500(27), 532sh | 376.0794 |
| Javanicin | $C_{15}H_{14}O_6$ | 14.46 | 228(100), 304(31), 480sh, 504(28), 538sh | 290.0790 |
| Lambertellin | $C_{14}H_8O_5$ | 14.75 | 212(100), 288(79), 432(21) | 256.0372 |
| Marticin | $C_{18}H_{16}O_9$ | 13.48 | 228(100), 304(33), 480sh, 500(29), 532sh | 376.0794 |
| Mitorubrinic acid | $C_{21}H_{16}O_9$ | 12.86 | 212(78), 272(100), 296sh, 448(65), 362sh, 393sh, 418sh, 448sh | 412.0794 |
| Mollisin | $C_{14}H_{10}Cl_2O_4$ | 21.35 | 208(100), 260(75), 420(11) | 311.9956 |
| Nectriafurone | $C_{15}H_{12}O_7$ | 13.48 | End(100), 240(81), 258(80), 324(25), 356sh, 444(57), 464sh | 304.0583 |
| Norjavanicin | $C_{14}H_{12}O_6$ | 11.78 | 212sh(72), 224(100), 298(37), 485sh(29), 498(30), 535sh | 276.0634 |
| Pachybasic acid | $C_{15}H_8O_5$ | 15.70 | 208(82), 224(80), 258(100), 278sh, 340(10), 404(20) | 268.0372 |
| Pachybasin | $C_{15}H_{10}O_3$ | 23.70 | End(94), 224(61), 248sh, 260(100), 277sh(47), 332(10), 404(20) | 238.0630 |
| Phoenicin | $C_{14}H_{10}O_6$ | 2.20 | End(78), 213sh(66), 268(100), 432broad(4) | 274.0477 |
| Phomarin | $C_{15}H_{10}O_4$ | 19.08 | 220(90), 268(100), 294sh, 415(21) | 254.2420 |
| Physcion | $C_{16}H_{12}O_5$ | 26.20 | End(60), 224(100), 257sh, 268(54), 288(55), 436(36) | 284.2683 |
| Puberulonic acid | $C_9H_4O_7$ | 1.32 | End(60), 276(100), 318(49), 363sh, 376(32), 412(30) | 223.9957 |
| Purpurin | $C_{14}H_8O_5$ | 17.06 | 204(72), 256(100), 296(30), 456sh, 480(28), 508sh | 256.0372 |
| Purpurogenone | $C_{29}H_{20}O_{11}$ | 21.56 | 220(100), 252(80), 273sh(50), 305(32), 364sh, 380(29), 490sh, 496sh, 520(27), 556sh | 544.1006 |
| Questin | $C_{16}H_{12}O_5$ | 16.22 | 224(100), 250sh, 284(64), 436(27) | 284.2683 |
| Questinol | $C_{16}H_{12}O_6$ | 9.39 | 224(100), 250sh, 284(64), 436(27) | 300.2677 |
| Ravenelin | $C_{15}H_{10}O_5$ | 19.02 | End(75), 236sh(60), 260(100), 340(37), 398(10) | 258.0528 |
| Riboflavin | $C_{17}H_{20}N_4O_6$ | 1.65 | 224(91), 268(100), 371(30), 448(41) | 376.1383 |
| Roseopurpurin | $C_{16}H_{12}O_6$ | 9.32 | End(74), 220(100), 248(54), 284(67), 436(28) | 300.0634 |
| Skyrin | $C_{30}H_{18}O_{10}$ | 24.96 | 224(100), 256(81), 296(52), 456(40) | 538.0900 |
| Solaniol | $C_{15}H_{16}O_6$ | 13.80 | End(60), 228(100), 308(30), 480sh, 504(28), 542sh | 292.0947 |
| Soranjidiol | $C_{15}H_{10}O_4$ | 19.93 | 220(90), 268(100), 293sh(50), 412(26) | 254.0579 |
| Stipitatic acid | $C_8H_6O_5$ | 1.36 | 266(100), 310sh, 360(20), 415sh | 182.0215 |
| 3,4,5-trihydroxy-7methoxy-2-methylanthraquinone | $C_{16}H_{12}O_6$ | 20.45 | End(70), 230(69), 260sh, 282(100), 313(43), 432(40) | 300.0634 |
| Viomellein | $C_{30}H_{24}O_{11}$ | 22.02 | 228(48), 275(100), 360(18), 380(18), 415(17) | 560.1319 |
| Viridicatumtoxin | $C_{30}H_{31}NO_{10}$ | 22.80 | 240(70), 284(100), 332(10), 436(30) | 565.1948 |
| Xanthomegnin | $C_{30}H_{22}O_{12}$ | 18.64 | 232(100), 288(30), 403(20) | 574.1111 |

# APPENDIX C

Supplementary information for Chapter 5.

Figure1. LC-DAD-ESI$^+$-MS chromatograms depicting reference metabolites citrinin and rubropunctamine used in the present study.

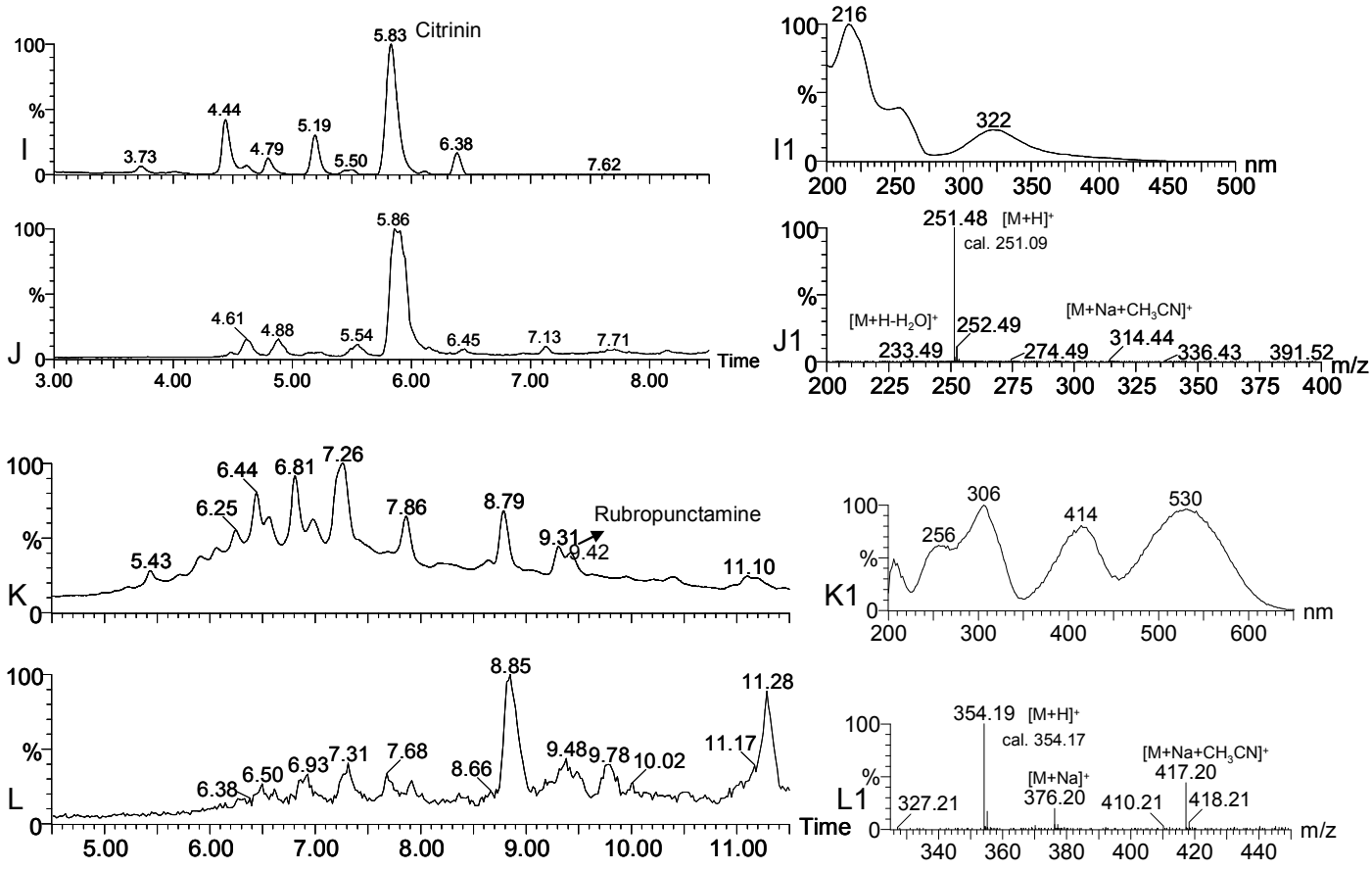


Figure 2. LC-DAD-ESI$^+$-MS chromatograms depicting new hits [X-hit no. 6 and 9] i) threonine derivative of rubropunctatin in *P. aculeatum* IBT 14263 on CYA, ii) monascorubrin in *P. pinophilum* IBT 13104 on YES medium.

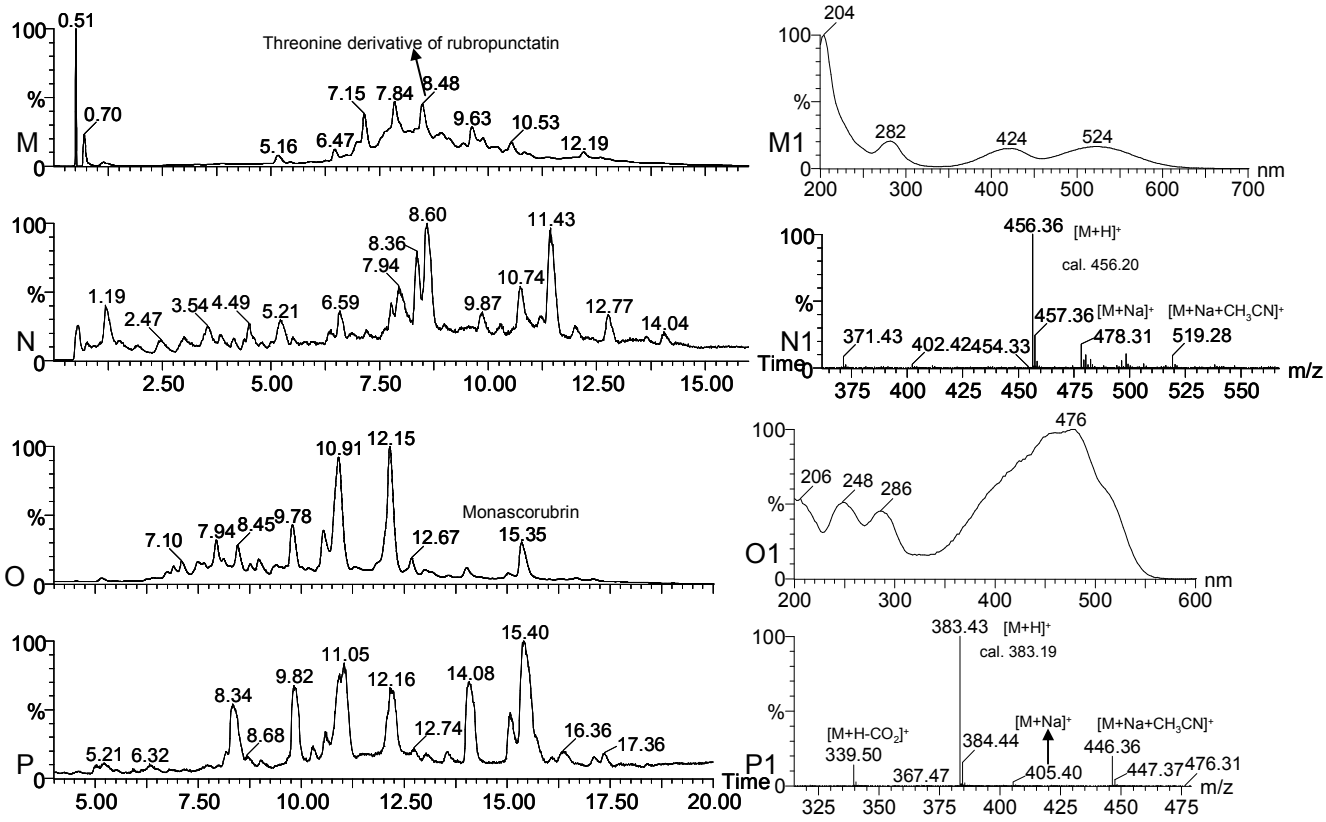


Figure 3. LC-DAD-ESI$^{+}$-MS chromatograms depicting new hit [X-hit no. 31] ankaflavin in *M. purpureus* IBT 9664 on YES medium.

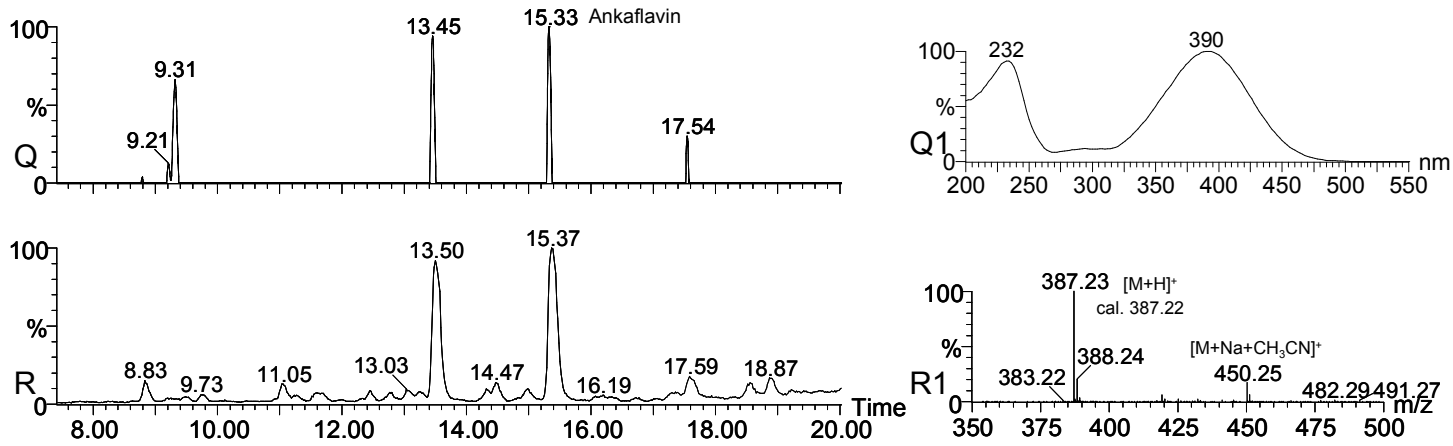
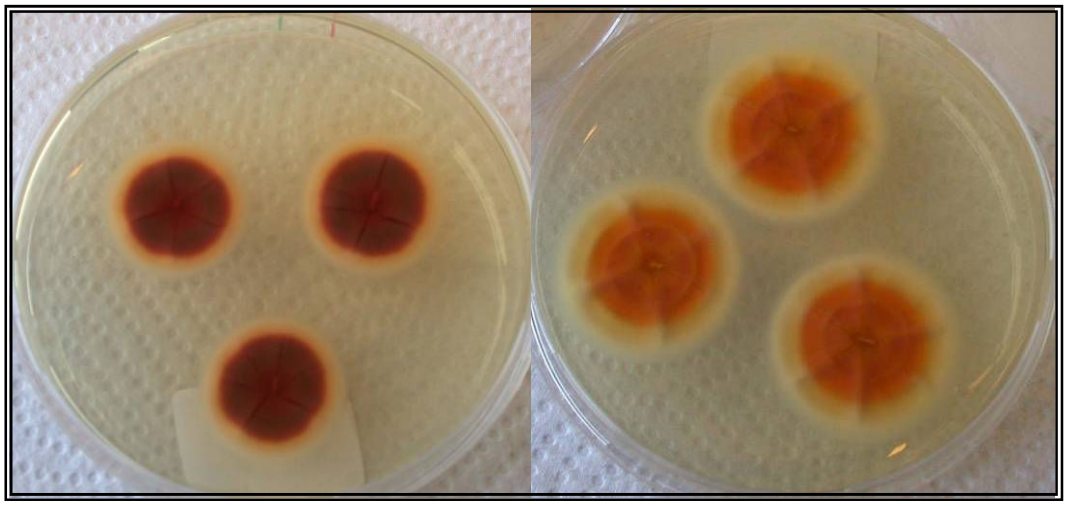


Figure 4. *Penicilliun aculeatum* IBT 14263 (left) and *Penicillium pinophilum* IBT 13104 (right) on YES media (Reverse showing the color produced by the mycelia)



# APPENDIX D

Patent Application WO 2009/026923 A2

**Production of Monascus-like azaphilone pigment**
Mapari, Sameer, A., S.; Meyer, Anne, S.; Frisvad, Jens, C.; Thrane, Ulf.

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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WO 2009/026923 A2

(54) Title: PRODUCTION OF MONASCUS-LIKE AZAPHILONE PIGMENT

(57) Abstract: The present invention relates to the field of biotechnological production of polyketide based colorants from filamentous fungi, in particular a method for preparing a biomass comprising a *Monascus*-like pigment composition from a nontoxigenic and non-pathogenic fungal source. The present invention further relates to use of the *Monascus*-like pigment composition as a colouring agent for food items and/or non-food items, and a cosmetic composition comprising the *Monascus*-like pigment composition.